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Delbert Mauritz Bergenstal

1917–1959

Late Assistant Chief

Endocrinology Branch

National Cancer Institute

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Delbert Bergenstal has gone from the world, a reality still difficult to grasp—just as it was difficult in life to appreciate fully the depth of his personality or the wealth of his talents.

Born in Kansas City, Missouri, in 1917, he received his college education at the University of Arkansas. He was awarded his Ph.D. degree in biochemistry from Purdue University in 1943, and his M.D. degree from the University of Chicago in 1947. After a residency in internal medicine at Presbyterian Hospital, New York, he returned to Chicago in 1949 as a Damon Runyon Clinical Research Fellow. In 1951, he organized and directed the arthritis clinic at the University of Chicago, where he remained until 1955, attaining the rank of Associate Professor of Medicine. Dr. Bergenstal received a commission in the Reserve Corps of the Public Health Service in 1955. At the time of his death he held the rank of Medical Director, and was Assistant Chief of the Endocrinology Branch of the National Cancer Institute. He was also active on several committees and panels of the Cancer Chemotherapy National Service Center of the National Cancer Institute.

These facts are the bare framework of his professional career. To them have been added professional distinctions, society memberships, prize awards, and positions of trust—all attesting to the high regard in which he was held by his colleagues in the medical profession.

Without detracting in any way from his accomplishments, Delbert Bergenstal was happiest and most effective when working with his coworkers, and an evaluation of his accomplishments proves that he was a great collaborator. Many of his associates were young medical students whom he was training. He contributed much to many programs, usually doing most of the work himself.

He was distinguished for his contributions to the following fields: 1. Some of the earlier studies on the metabolic influence of ACTH and excesses of cortisone. The characterization of the catabolic processes induced by adrenal and thyroid hormones. 2. The clinical management of adrenalectomy in man. 3. The effect of adrenalectomy on advanced breast and prostatic cancer. 4. The use of yttrium^{90} beads for the destruction of the hypophysis in man, as a means of favorably influencing the growth of metastatic breast cancer. 5. Studies on the metabolic influence of synthetic steroids with corticoid and progestational activity. 6. The demonstration

that progesterone is a catabolic and natruretic hormone. 7. The securing of evidence that prolactin may be protein-anabolic in hypopituitary individuals. 8. Demonstration that the uptake of radioactive sulfur by cartilage could be used as a means of quantitating hormonal action on the growth of cartilage tissue—in particular pituitary growth hormone. 9. Studies on the influence of DDD on adrenal cancer.

Delbert first joined my Laboratory when he was an undergraduate student of medicine, and his first work was the isolation of steroid hormones from urine. He worked prodigiously and effectively and in an instant earned the affection of all his coworkers. Our association was very close for 10 years. During the period when it was found that the adrenal glands could sustain mammary cancer in human beings, we had conversations lasting at least an hour a day—practically every day of the year. Always, he gave the news of his family first, and then of his work with patients and of any new discoveries; there was always talk of hormones, cancer, and endocrinology.

Del was a superb clinician with great ability in clinical investigation. He had a sunny personality, which attracted friends who were magnetized to him by strong bonds of devotion. He was a sympathetic physician who was adored by his patients. He married Alice Shriener; with their sons, Karl and Richard, they were a close-knit family unit.

Early in 1957, it was discovered that he was suffering from an advanced hepatic cirrhosis, possibly related to his long contact with organic solvents in his scientific work. He bore with equanimity the knowledge that he was suffering from a fatal disease. He never complained—he only worked harder and with even greater devotion to alleviating the plight of patients with cancer. Death came on September 12. Thus ended a brilliant career which already had permanent impact on his chosen field of clinical endocrinology and seemed destined to go on to even higher levels of achievement.

Charles Huggins

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Cytochemical Studies of Milk-Adapted Murine Lymphoma Cells (Strain P388 D_1) Infected With Polyoma Virus[1]

ROBERT LOVE *and* ALAN S. RABSON,[2] *Laboratory of Pathology, National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

Some aspects of the cytochemistry of milk-adapted murine lymphoma cells are presented. Infection of the cells with polyoma virus produces an alteration in the ribonucleoprotein (RNP) of the parachromatin of the nucleolus and nucleoplasm, and enlargement of the nucleus, nucleolus, and cytoplasm; vacuoles containing an abnormal RNP appear in the nucleus. Cell degeneration is completed by nuclear and cytoplasmic shrinkage, disappearance of cytoplasmic RNP, and karyorrhexis. It is suggested that the mechanisms of synthesis of normal RNP in the nucleus may be diverted to the production of RNP that is related to viral synthesis.—J. Nat. Cancer Inst. 23: 875–891, 1959.

MANY VIRUSES are capable of inducing necrosis, cellular proliferation, or both (*1*, *2*). The polyoma virus is of unusual interest because it produces a variety of proliferative lesions in several species (*3*). In addition, a cytopathogenic effect has been observed in cell cultures infected with this virus (*4*). The present study was designed to investigate the cytopathology and, particularly, the changes in nucleoproteins in tissue-culture cells infected with polyoma virus. Mouse lymphoma cells (strain P388 D_1) grown in a serum-free milk medium were selected because they constitute a relatively uniform cell population and because a rapid, predictable cytopathogenic effect occurs after inoculation of the polyoma virus (*5*).

MATERIALS AND METHODS

Cell cultures.—Cell strain P388 D_1 was established in culture by Dawe and Potter from the 49th mouse passage of a methylcholanthrene-induced lymphoid neoplasm of a DBA/2 mouse (*6*). The strain was originally grown in a medium containing 40 percent human serum and was later adapted to grow in a serum-free medium containing 20 percent auto-

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claved "nonfat milk" and 80 percent mixture #199. The adaptation of these cells to the nonfat milk medium, the preparation of the medium, and the methods of growing the milk-adapted cells have been described previously (*7*).

In the present experiments, cultures of milk-adapted P388 D_1 cells were grown on coverslips in Leighton tubes, for cytochemical studies. Approximately 800,000 cells suspended in 2 ml. of milk medium were placed in each tube and incubated in stationary racks at 36.5° C. for 1 hour to permit the cells to adhere to the coverslip. Cultures were then inoculated with 0.25 ml. of the virus inoculum and incubated again at 36.5° C. Control cultures were inoculated with supernatant fluid from uninfected cultures.

Virus.—The strain of polyoma virus used in these studies was initially isolated in cultures of milk-adapted P388 D_1 cells from a cell-free filtrate of a parotid-gland tumor extract from a C3H/Bi mouse. The derivation of this strain of virus will be described by Dawe and his associates. The cytopathogenic effect produced by this virus in milk-adapted P388 D_1 cells, the serial passage of the virus in these cultures, and the lesions produced in hamsters after inoculation with virus grown in culture have been described (*5*).

Virus from the thirteenth, fourteenth, and fifteenth serial passages in milk-adapted P388 D_1 cells was used in the present studies. All virus preparations were stored in glass tubes with rubber stoppers at −20° C. in an electric freezer. The titer of the thirteenth- and fifteenth-passage material was not determined. The pool of fourteenth-passage material was titrated by serial tenfold dilutions in tube cultures of milk-adapted P388 D_1 cells; it contained $10^{6.5}$ tissue-culture infectious doses (TCID50) per 0.25 ml. Cultures were inoculated routinely with undiluted virus preparations. Since some of the changes in the cultures inoculated with virus might be due to the toxic effect of the inoculum rather than to multiplication of the virus (*8*), a 10^{-4} dilution of the fourteenth-passage pool was used to produce a delayed cytopathogenic effect.

A group of P388 D_1 cultures was also inoculated with a pool of the seventh and eighth mouse-embryo tissue-culture passage of LID-1 strain of polyoma virus, pool #358 (*9*), provided by Dr. W. P. Rowe, Laboratory of Infectious Diseases, National Institutes of Health.

Neutralization test.—A neutralization test was performed with the fourteenth-passage virus preparation and polyoma virus antiserum obtained from Dr. Rowe. The antiserum was prepared by immunizing weanling mice with mouse-embryo tissue-culture fluid infected with polyoma virus. Control mouse serum was obtained from mice immunized with mouse-embryo tissue-culture fluid free of polyoma virus. A sample of virus containing approximately 3,000 TCID50 was mixed with an equal volume of a 1:10 dilution of the mouse antiserum, and the mixture was incubated at room temperature for 1 hour before inoculation of tube cultures of milk-adapted P388 D_1 cells. The same procedure was repeated with the control mouse serum.

Cytological techniques.—Cultures were examined between 48 and 72 hours after inoculation of undiluted virus and 9 days after introduction of diluted virus. In most instances the medium was removed and the cells were fixed directly in the Leighton tubes. Although the milk in the medium was low in fat content, it was necessary to wash the cells with physiological saline, before fixation, for the demonstration of lipides. Living cells were also examined under the phase microscope, in the medium and in physiological saline.

Nucleoproteins were stained by the toluidine blue-molybdate (TBM) procedure (*10*, *11*), by pyronin-methyl green (*12*), and by the Feulgen method (*12*). Cultures were also prepared and stained as described for the TBM method, with the omission of the molybdate coupling. Stages I and II of staining by the TBM method were produced by fixation for 5 and 90 minutes, respectively, in formol sublimate at 37° C. Cultures were fixed for 60 minutes and deaminated overnight in nitrous acid (*11*) to produce stage III of staining. Because some forms of ribonucleoprotein (RNP) in the virus-infected cells were unstained by these procedures, cultures were also fixed for 3, 30, 45, 90, and 120 minutes. The concentrations of toluidine blue and molybdate were the same as those for smears of ascites tumor cells (*11*). The specificity of the staining procedures for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was checked by observing the effect of digestion with ribonuclease and extraction of both nucleic acids with hot trichloroacetic acid (*10*, *11*).

Various protein-bound groups were demonstrated after brief fixation (15 minutes) in formol sublimate at 25° C. by the dinitrofluorobenzene (DNFB) and ninhydrin-Schiff procedures (*13*). Similarly, fixed cultures were stained with Schiff's reagent and by the periodic acid-Schiff (PAS) method (*14*), with and without digestion of glycogen by diastase (*12*) and extraction of lipides with boiling chloroform-methanol. Lipides were stained with Sudan black B after fixation for 1 hour in formol sublimate at 37° C. Cultures were fixed for 6 hours in formol sublimate containing 1 percent calcium chloride, and stained by Baker's acid hematin method for phospholipides (*15*). A few cultures were fixed in Bouin's fluid and stained with hematoxylin and eosin.

RESULTS

Uninfected P388 D_1 Cells

Three different staining patterns of nucleoproteins were produced by the TBM method (table 1). After removal of nucleic acids by extraction with hot trichloroacetic acid, cells could not be stained by the TBM procedure.[4] With the exception of very pale-green coloration of DNA, all staining in stages I and II (table 1) was due to the presence of RNP and was prevented by digestion with ribonuclease. In stage III prepara-

[4] In stage III preparations, occasional granules of protein in the cytoplasm were pale green after extraction with trichloroacetic acid.

tions, the cytoplasm and nucleolus did not stain after ribonuclease digestion, and the intensity of staining of chromatin was reduced. Since the chromatin did not stain after extraction of both nucleic acids, both DNP and RNP (chromosomal RNP) were responsible for chromatin staining in stage III preparations (table 1).

Since the distribution of the various forms of RNP during mitosis was similar to that described in other dividing cells (*10, 11*), only the interphase cell will be described.

Stage I (*table 1*).—Finely granular and amorphous metachromatic RNP (parachromatin) was scattered throughout the nuclear sap and formed one or more small purple granules in the green nucleolus (fig. 1). Minute, unstained vacuoles were detected in most of the larger nucleoli and, not uncommonly, the metachromatic nucleolar parachromatin formed a partial or complete membrane around the periphery of the vacuoles. The diffuse cytoplasmic RNP was stained uniformly green, except in the Golgi zone where the intensity of staining was less and there was much coarse vacuolation (fig. 1). Occasional micronuclei were present in the cytoplasm and multinucleated giant cells were not infrequent.

Stage II (*table 1*).—The parachromatin was no longer stained and the nucleolar and diffuse cytoplasmic RNP were pale green (fig. 2). The perichromosomal and cytoplasmic granular RNP was stained purple (fig. 2). The distribution of perichromosomal RNP was similar to that of DNA, but more diffuse (*cf.* figs. 2 and 8).[5]

Stage III (*table 1*).—The parachromatin, perichromosomal, and cytoplasmic granular RNP were no longer detectable (fig. 3). The diffuse cytoplasmic RNP and the nucleolus were stained purple, and also the DNP and RNP (chromosomal RNP, table 1) of the chromatin. A few unstained or pale-green granules that were unaffected by extraction of nucleic acids were present in the cytoplasm of occasional cells.

When molybdate coupling was omitted, P388 D_1 cells were stained by toluidine blue in the same manner as other neoplastic cells (*11*). Fixation and deamination produced a progressive increase in binding of dye by the cytoplasm, nucleolus, and the chromatin. The pyronin-methyl green stain colored the cytoplasm and nucleolus red and the chromatin green; the parachromatin, cytoplasmic granular, and perichromosomal RNP were not detectably stained.

The unstained or pale-green granules that were seen in the cytoplasm of occasional cells in stage III TBM preparations were stained by the dinitrofluorobenzene and ninhydrin-Schiff procedures. Protein-bound groups were also revealed by these stains in the nucleolus, the chromatin, and throughout the cytoplasm; minute granules and faint, diffuse-staining material were present among the unstained vacuoles in the Golgi zone. The direct Schiff reaction was negative, and abundant granular and diffusely staining diastase-resistant PAS-positive material was found among large, unstained vacuoles in the juxtanuclear area of the cyto-

[5] The relationship of the perichromosomal RNP to the chromatin was more readily apparent in metaphase in which the metachromatic RNP formed a well-defined sheath around the light-green chromosomes (*11*).

TABLE 1.—Staining reactions of ribonucleoproteins (RNP) by the toluidine blue-molybdate method in uninfected and polyoma virus-infected P338 D_1 cells*

Stage of staining produced	Fixation and deamination	Presence or absence of polyoma virus	Nucleoli (*pars amorpha*)	Parachromatin of nucleolus and nuclear sap	Perichromosomal and cytoplasmic granular RNP	Diffuse cytoplasmic RNP	Chromosomal RNP	Content of nuclear vacuoles
I	5 minutes formol sublimate 37° C.	Uninfected	Green ++	Purple +	NR†	Green ++	NR	Absent
		Infected	Green ++ enlarged	NR	NR	Green ++‡	NR	Trace green
II	90 minutes formol sublimate 37° C.	Uninfected	Green +	NR	Purple +	Green +	NR	Absent
		Infected	Green + enlarged	NR	Purple +‡	Green +	NR	Trace green
III	60 minutes formol sublimate + 18 hours nitrous acid	Uninfected	Purple++++	NR	NR	Purple +++	Purple +	Absent
		Infected	Purple++++ enlarged	NR	NR	Purple +++	Purple +	Purple + to ++++§

*The final stages of cell degeneration characterized by extreme pyknosis and karyorrhexis are described in the text.

†NR: Not recognizably stained.

‡Staining of RNP in these sites was reduced in enlarged cells and normal after moderate shrinkage.

§+ to ++++ indicates intensity of staining.

plasm. Most of this PAS-positive substance was extracted by chloroform-methanol, leaving small amounts of diffuse and finely granular PAS-positive material in the Golgi zone. Since the latter resembled the protein demonstrated by the DNFB and ninhydrin-Schiff methods, it was probably glycoprotein. Large, intensely sudanophilic droplets were present in the Golgi area and occasionally elsewhere in the cytoplasm; these appeared to correspond to the unstained vacuoles in the PAS preparations. In addition, finely granular and diffuse, less intensely sudanophilic lipide was also present in the Golgi zone; this was probably the PAS-positive lipide that was extracted by chloroform-methanol. Similar material was stained by Baker's acid hematin method for phospholipide. The mitochondria were not recognizably stained by any cytochemical technique.

Examination of living cells under the phase microscopic revealed an almost structureless nucleus containing several prominent nucleoli and very numerous fine granules in the juxtanuclear area of the cytoplasm (fig. 10).

A few degenerating cells were present in the uninfected cultures, and, in preparations where degeneration was most conspicuous, fragments of nucleoprotein, probably derived from nuclear debris, were found in the cytoplasm of many cells. Degeneration was characterized by regular shrinkage of the nucleus and homogeneous metachromatic staining of the nuclear RNP by the TBM method. In cells with moderate-to-severe pyknosis, the cell body was rounded and shrunken, and the cytoplasmic granular and diffuse RNP did not stain. There was no consistent change in the cell lipides or the PAS-positive material in the Golgi zone. Cell destruction was completed by fragmentation of the nucleolus, dissolution of the cytoplasm, and karyorrhexis.

P388 D_1 Cells Infected With Polyoma Virus

Extensive cytopathogenic changes and all gradations from normal to disintegrating cells were present 48 hours after inoculation of undiluted virus. The cytopathogenic effect produced by the LID-1 strain of virus was less extensive but essentially the same as that in cultures inoculated with the strain that had been carried in P388 D_1 cells. Degenerating cells were scattered irregularly throughout the cultures and there was no suggestion of focal plaque formation. Giant cells were no more frequent than in the uninfected cultures. An increasing number of abnormal [6] cells appeared between 48 and 72 hours after inoculation of virus, many of the cells were detached from the glass, and virtually no normal [6] cells remained. The changes produced by dilute virus were identical but did not appear until 9 days after infection. In the neutralization test, a cytopathogenic effect was observed 7 days after inoculation of the virus-

[6] The terms "abnormal" and "normal" in reference to the cells and to their contents are used relatively and do not imply that P388 D_1 cells, which were derived from a neoplasm, are normal in any respect.

control serum mixture. The cytopathogenic effect was delayed until 13 days in the cultures inoculated with the virus-antiserum mixture. Although clumping of chromosomes was occasionally observed in metaphase and anaphase, degenerative changes were almost entirely confined to cells in interphase. A conspicuous decrease in the number of mitotic figures suggested that, for the most part, the infected cells were incapable of mitotic division.

With the TBM stain, a striking series of changes in RNP was demonstrated in the virus-infected cells. The earliest abnormality consisted of nuclear, nucleolar, and cytoplasmic enlargement, rounding of the cell body, and disappearance of the stainable parachromatin, perichromosomal, and cytoplasmic granular RNP (figs. 1 through 7, and table 1); the intensity of staining of the diffuse cytoplasmic RNP was decreased (table 1). One or more small unstained vacuoles were seen in the majority of enlarged nuclei (fig. 4). The parachromatin, perichromosomal, and cytoplasmic granular RNP in the enlarged virus-infected cells could not be stained in preparations fixed for 3, 5, 30, 45, 60, 90, and 120 minutes. Since occasional normal cells in the infected cultures were well stained, failure to stain the various ribonucleoproteins was not due to changes in the medium. Further disorganization of the cell was characterized by progressive enlargement of the nuclear vacuoles and moderate regular shrinkage of the nucleus. At this stage an abnormal RNP was detected in the vacuoles. This material was digested by ribonuclease and was colored very pale green by TBM in stages I and II, and purple in stage III (figs. 5, 6, and 7, and table 1). In the smaller vacuoles the abnormal RNP was homogeneous and weakly stained; the larger vacuoles were filled with the faintly staining material but also contained one or more intensely staining, sometimes vacuolated bodies (figs. 6 and 7). The contents of some larger vacuoles were very weakly stained by pyronin and by toluidine blue, without the use of molybdate. Weak but definite staining by the DNFB and ninhydrin-Schiff procedures confirmed the presence of protein in association with RNA in the vacuoles. In hematoxylin and eosin preparations, the contents of the vacuoles were stained by eosin (*5*), presumably because of the basic amino groups of the protein component of the RNP. Unstained dense bodies in the vacuoles could be seen with the phase and even with the direct light microscope in Feulgen preparations (figs. 8 and 9); less commonly, minute granules of DNA were detected in some of the vacuoles. The vacuoles and dense bodies inside them were readily seen in living cells examined under the phase microscope (figs. 11 and 12).

Accumulation of abnormal RNP in the vacuoles was accompanied by shrinkage of the nucleus and, to a lesser extent, of the cytoplasm. By the time the abnormal nuclei had shrunk to the size of the normal nuclei, staining of the perichromosomal RNP (but not the parachromatin) was unimpaired (fig. 5). In some shrunken cells the cytoplasmic granular RNP was also stained and the intensity of coloration of the diffuse cytoplasmic RNP was not reduced. The impairment of staining of these forms of RNP in the swollen cells may therefore be the result of dilution rather

than chemical change. The cytoplasmic granular and diffuse RNP were no longer detectable in the most shrunken cells with minute pyknotic nuclei. The parachromatin did not stain except in the terminal phase of nuclear degeneration when the small pyknotic nuclei or nuclear fragments were stained uniformly purple. Both RNP and DNP were stained metachromatically since the metachromasia was reduced but not abolished by ribonuclease digestion, and the pyknotic nuclei did not stain after extraction with trichloroacetic acid. The chromosomal RNP, as determined by the reduction in the intensity of chromatin staining produced by ribonuclease in stage III TBM preparations, was not perceptibly altered in the virus-infected cells (table 1).

There was an increase in the number and size of the large, intensely sudanophilic lipide droplets in the virus-infected cells. The PAS-positive phospholipide and protein components of the Golgi zone were unchanged. Granules of protein, like those in the uninfected cells, were present in the cytoplasm of some cells. When the disintegration of cells became widespread, increasing numbers of nucleoprotein granules derived from fragmentation of nuclei were found inside and outside cells.

DISCUSSION

Differential staining of nucleoproteins by the TBM method depends upon variation in the susceptibility of the amino groups of nucleoproteins to inactivation by formaldehyde or nitrous acid; this, in turn, determines the amount of dye bound and the type of complex formed between the nucleic acid-phosphoryl group and the dye cations (*11*). Each nucleoprotein requires a certain amount of fixation or deamination to permit the formation of the type of nucleic acid-dye complex that will react with molybdate to produce intense metachromatic staining. Some nucleoproteins, like the parachromatin, bind basic dyes so weakly that they can only be visualized by the intensification of staining produced by molybdate (*11*). The parachromatin of the virus-infected cells did not stain after the amount of fixation required to stain the parachromatin in cells of control cultures. The absence of parachromatin staining in the enlarged nuclei of infected cells could be the result of dilution associated with an increase in nuclear volume; this could not, however, account for the absence of demonstrable parachromatin in normal-sized and shrunken nuclei. Changes in the *p*H or in other constituents of the cell or the medium or variations in cell permeability might alter the fixation time required to produce metachromatic staining of the parachromatin. The parachromatin in the infected cells did not stain even after a wide range of fixation times. The evidence suggests that infection with polyoma virus produced some qualitative and perhaps quantitative changes in the RNP of the parachromatin.

Simultaneously with, or shortly after, the changes in parachromatin, vacuoles devoid of stainable material appeared in the nucleus. Still later,

abnormal RNP was formed in the vacuoles. The mechanisms of synthesis of normal RNP in the nucleus may therefore be diverted to the production of RNP that is concerned with the synthesis of virus. The chemical nature of the polyoma virus is not known, but there is considerable evidence that RNA may carry the genetic template of a number of viruses (*16–18*) including those which produce tumors (*19*).

Little is known about qualitative changes in RNA metabolism in virus-infected cells. In Ehrlich ascites tumor cells infected with a strain of Newcastle disease virus (NDV), there appears to be a synthesis of RNP resembling the parachromatin in the nucleus; this substance is extruded into the cytoplasm of the Golgi region where it is probably incorporated into phospholipide-covered inclusions (*20*). No qualitative differences could be detected between the nuclear RNP of uninfected and infected Ehrlich cells. Nucleolar enlargement was a common feature of infection with NDV and polyoma virus; with the former, the nucleolar parachromatin was unusually conspicuous, whereas in polyoma-virus infection the parachromatin was apparently deficient. In a study of the cytochemistry of chick-embryo liver tissue cultures infected with RPL-12 lymphomatosis virus, Defendi and Sharpless observed the formation of Feulgen-positive intranuclear inclusions (*21*). Rabotti (*22*) analyzed by microspectrophotometry the DNA content of P388 D_1 cells infected with polyoma virus and failed to find any increase in DNA. The cytochemical observations presented here also provide no evidence of increased synthesis of DNA. There is insufficient information at present to generalize about differences between the cytopathogenic effects of viruses that induce proliferation or neoplasia and those that do not.

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PLATES

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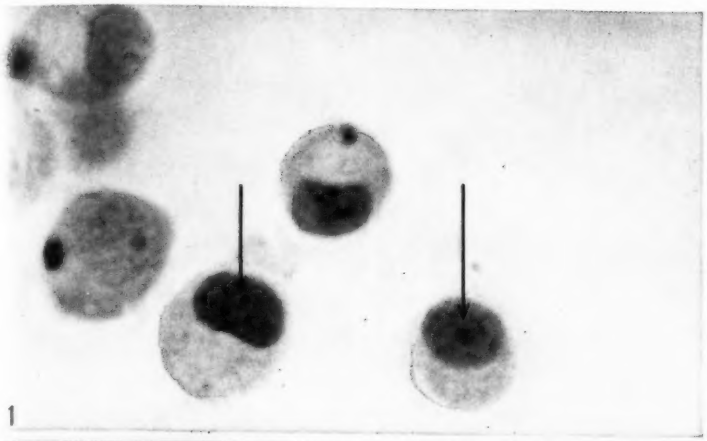
PLATE 73

FIGURE 1.—P388 D_1 cells stained by the toluidine blue-molybdate (TBM) method, stage I. *Note* purple parachromatin in the nucleoli (*arrows*) and in the nucleoplasm. The *pars amorpha* of the nucleolus is green. The cytoplasm is coarsely vacuolated in the Golgi zone. Uninfected P388 D_1 cells may be rounded as in this figure, or fusiform as in figures 2 and 3. × 1,600

FIGURE 2.—P388 D_1 cells stained by TBM, stage II. The diffuse cytoplasmic RNP is pale green and the cytoplasmic granular and perichromosomal (P) RNP are purple. The nucleolus (N) is green. × 1,600

FIGURE 3.—P388 D_1 cells stained by TBM, stage III. The chromatin and the nucleoli are dark purple. The diffuse cytoplasmic RNP is less intensely stained. × 1,600



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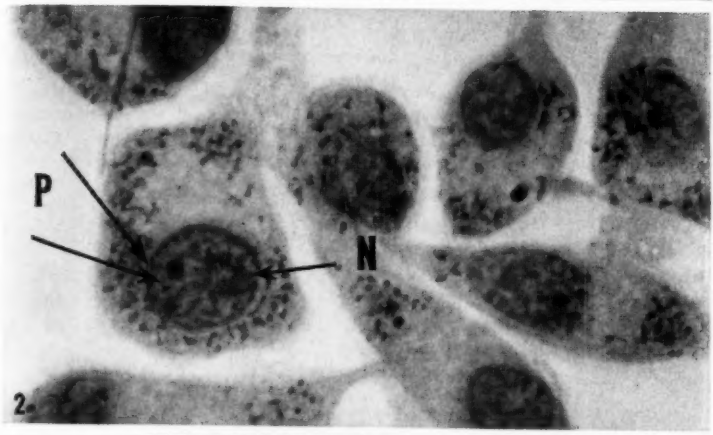

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PLATE 74

FIGURE 4.—P388 D_1 cells infected with polyoma virus, stained by TBM, stage I (*cf.* fig. 1). Metachromatic staining of parachromatin is impaired and there are unstained vacuoles (*arrows*) in some of the enlarged nuclei. × 1,600

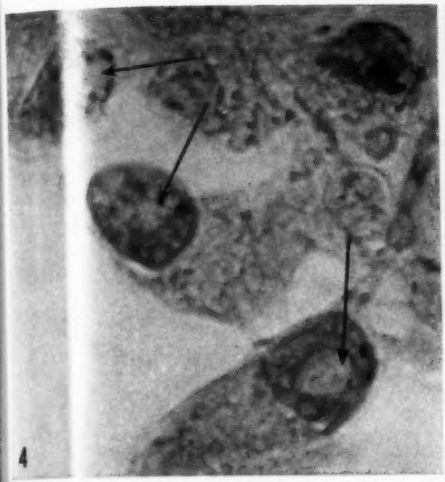
FIGURE 5.—P388 D_1 cells infected with polyoma virus, stained by TBM, stage II (*cf.* fig. 2). The cytoplasmic granular RNP is unstained; the perichromosomal RNP of the slightly shrunken nucleus is stained purple. The green-staining nucleolus (N) is normal in size and there is a pale green-colored body (B) inside a small vacuole. × 1,600

FIGURE 6.—P388 D_1 cells infected with polyoma virus, stained by TBM, stage III (*cf.* fig. 3). The nucleus, nucleolus, and cell body are enlarged. A vacuole in the nucleus of one cell contains diffuse purple-staining RNP. In the center of the vacuole a more intensely staining body (*arrow*) is present. × 1,600

FIGURE 7.—A P388 D_1 cell infected with polyoma virus, stained by TBM, stage III. The same changes are present as in figure 6 except that fine vacuoles are in the large intensely stained RNP body (*arrow*) in the nucleus. × 1,600

FIGURE 8.—P388 D_1 cells stained by the Feulgen method. × 1,600

FIGURE 9.—A P388 D_1 cell infected with polyoma virus, stained by the Feulgen method. *Note* opaque Feulgen-negative bodies (*arrows*) in the nuclear vacuoles (*cf.* fig. 8). × 1,600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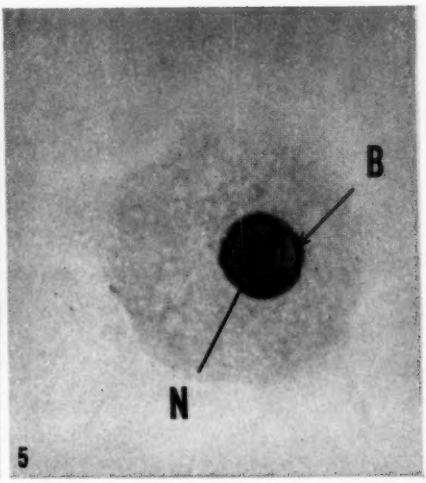

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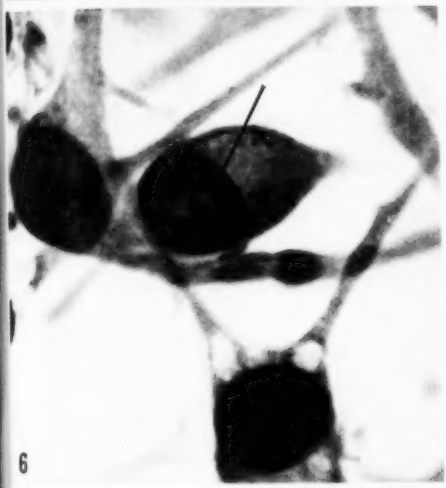
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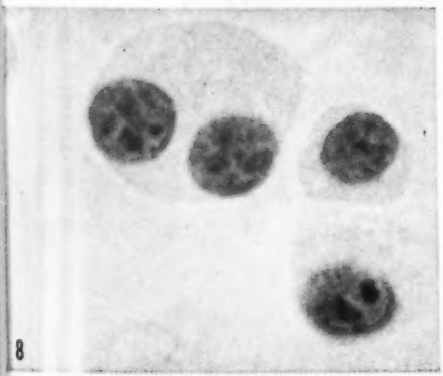
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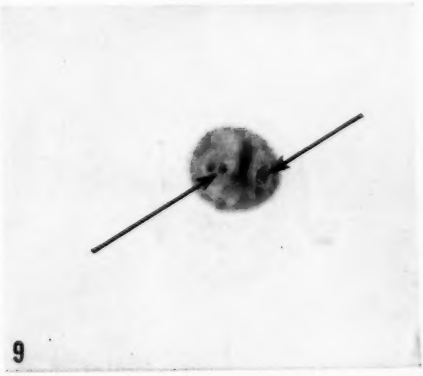
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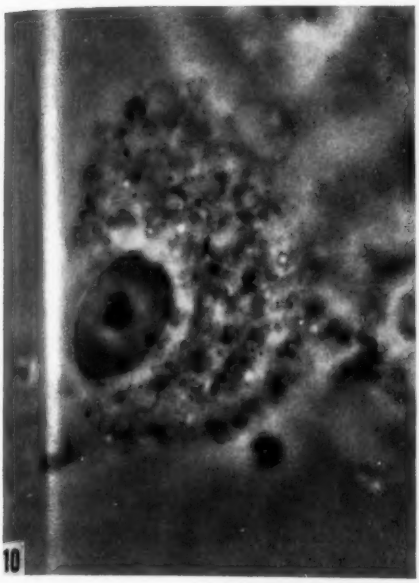
PLATE 75

FIGURE 10.—Phase-contrast photograph of a P388 D_1 cell. *Note* large nucleolus in the relatively structureless nucleus. The cytoplasm contains numerous granules, especially in the Golgi zone. × 1,400

FIGURE 11.—Phase-contrast photograph of a P388 D_1 cell infected with polyoma virus. *Note* dense, round, enlarged nucleoli. A large irregularly shaped finely vacuolated body (*arrow*) is also present in the nucleus. × 1,400

FIGURE 12.—Phase-contrast photograph of a binucleate P388 D_1 cell infected with polyoma virus. In addition to the enlarged nucleoli each nucleus contains at least one large, dense body (B). (The bodies shown in this photograph are the largest that have been seen.) × 1,400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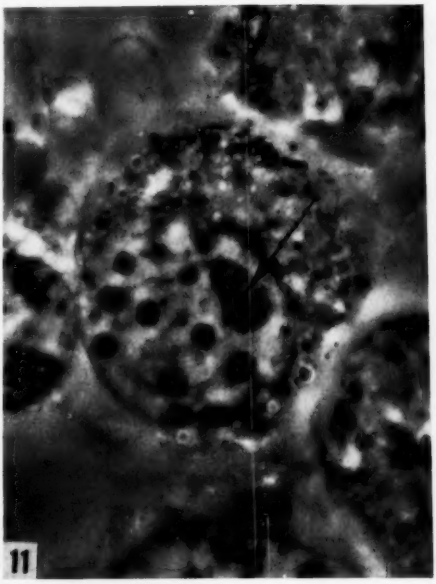
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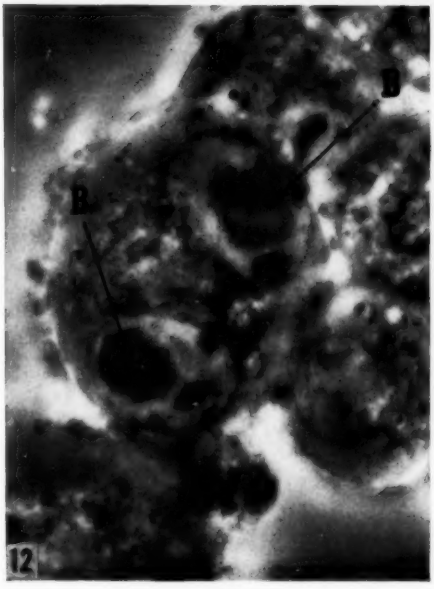
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Ultrastructure of Primary Culture Amnion Cells and Transformed FL Cells in Continuous Culture [1, 2]

JØRGEN FOGH *and* GEORGE A. EDWARDS, *Division of Laboratories and Research, New York State Department of Health, Albany, New York*

SUMMARY

Human amnion cells in primary culture and transformed amnion cells (strain FL) in continuous culture differ considerably in morphology when observed in thin sections with the electron microscope. Primary amnion cells are characterized by organized layers of organelles and bands of fine filaments in the cytoplasmic matrix. FL cells of young cultures contain uniform, small free vesicles within multivesicular bodies, hypertrophied Golgi complex, large mitochondria, and abundant ergastoplasm. FL cells of older cultures contain a cytoplasm abundantly filled with small mitochondria, unorganized endoplasmic reticulum, and dispersed Golgi zones. While multivesicular bodies are rare in older cultures, isolated, dense, membrane-limited particles, and concentric-membraned vesicular bodies, of several different morphologic types, are commonly observed in the cytoplasm and at the cell border. The multivesicular bodies could be either cellular reactions related to the malignancy factors of the FL cells or normal cellular components related to rapid growth. The membrane-limited particles of the older FL cultures are most logically assumed to be related to the transformation of normal human amnion cells into malignant cells.—J. Nat. Cancer Inst. 23: 893–923, 1959.

THE DISTINCTION between normal and malignant cells in culture, on the basis of their morphology and biochemistry, has so far been relatively inconclusive. Quantitative differences have been described, but until the present no specific structure such as the virus particles known to cause the development of certain animal tumors has been identified in human malignant cells in culture or in transplant, though structures of a virus-like morphology have been observed in a cervical lymph node in human acute leukemia (*1*).

The present paper presents comparative electron microscopic observations of tissue cultures of normal primary human amnion cells (*2*), and of transformed human amnion cells, strain FL (*3*). The latter cells have been characterized as malignant (*4–6*).

In a previous publication (*7*) the micromorphology of the intact normal human amniotic membrane and the effect of a short trypsinization on

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the cells therefrom have been described. Thus, it is now possible to compare several stages in this transformation system, from the cells of the original amniotic membrane through to the transformed and malignant FL cells. It is found that each of these stages has certain significant characteristics. The most striking feature of the changed cells (FL) is the presence of particles which vary in appearance with the growth stage of the transformed cultures. Such particles were not observed in the nonmalignant stages, nor have they been observed in other normal tissue-culture cells examined in our laboratory.

MATERIAL AND METHODS

Three human amniotic membranes, less than 1 hour after delivery, were each trypsinized for 2 hours in 0.25-percent trypsin (Difco) in Hanks' solution and centrifuged at 1000 rpm for 10 minutes. The cells from each membrane were seeded in tissue-culture tubes, with screw caps (Kimble 45107), containing coverslips, in a medium consisting of 2 parts human serum and 8 parts Hanks' solution to which had been added lactalbumin hydrolysate 0.5 percent, glutamine 2.5 mg. percent, valine 1 mg. percent, and glucose 0.1 percent. As a control on the influence of medium upon structure, several cultures were grown in LY-medium (*3*) containing 20 percent human serum. The respective primary cultures from the 3 amniotic membranes were prepared for electron microscopy after 9 and 11 days of growth, having been through one change of fluid.

Transformed human amnion cells, strain FL, subcultured continuously for 2½ years, were seeded in similar tubes with coverslips. In the transfer, Versene was used (Disodium versenate, Versenes, Inc.) at a concentration of 1:5000 in calcium- and magnesium-free phosphate buffer solution. The growth medium was LY-medium (*3*) containing 20 percent human serum. The cultures used in the present study were 3- and 6-day growths from the 126th transfer and 3- and 7-day growths from the 128th transfer, with fluid changed on the 4th day.

The coverslip cultures were left in their respective tubes during the preparation for electron microscopy until the final step of embedding. The material was washed in several changes of veronal-acetate buffer at *p*H 7.4, then fixed with OsO_4 (2% in veronal acetate buffered to *p*H 7.4) for 45 minutes. It was washed again with several changes of buffer for 10 minutes, dehydrated in 5-minute steps in 30, 50, 70, 90, and 100 percent ethanols, infiltrated with a dried, evacuated 1:1 mixture of ethanol and methacrylates (9 parts butyl to 1 part methyl) for 30 minutes, followed by 1½ hours in changes of methacrylates and a further 1½ hours in the methacrylate mixture containing 1½ percent Lucidol. The coverslips were then removed from the tubes with forceps and placed on glass slides wet with methacrylate; a clean cut was made through one end of the culture with a sharp razor blade (new blade for each prepara-

tion), and then the culture was rolled off, with the blade held at right angles to the coverslip. The resulting sheet of cells was divided into 2 to 4 sheets with the blade, and the pieces embedded in 00 gelatin capsules and polymerized for 36 hours at 46° C. in methacrylates plus Lucidol. This method of embedding has the advantage that as the rolled sheet of cells drifts down through the plastic in the capsule it gradually unfurls and comes to lie partly against the side and partly across the tip of the capsule, making it possible to cut parallel and normal sections of the cell sheet from a single block. Sections were cut with diamond knives on Porter-Blum microtomes, mounted on grids covered with Formvar lightly stabilized with carbon, and observed in a Siemens' Elmiskop I at initial magnifications of 1,000× to 20,000×.

RESULTS

a. Primary Cultures of Amnion Cells

The cells of the primary cultures grow on the coverslip as continuous monolayers. In sections normal to the plane of growth the cells appear generally flat and long, but occasional cells are more rounded and somewhat larger than the others. As the cells of both sizes and from both types of media possess a similar morphology, they will be described as one.

The border of the amnion cell is essentially smooth in outline but occasionally shows pseudopod-like projections (fig. 1) or small irregularly spaced finger-like projections (fig. 2). The cell is limited by a plasma membrane only; no basement membrane material or intercellular bars (desmosomes) are present. The cytoplasm of the cell is fairly well divided into three discrete layers of organelles. The peripheral cytoplasm contains Palade granules and both smooth and rough vesicular and tubular profiles of the endoplasmic reticulum (figs. 1, 2, and 3). Pinocytotic vesiculation of the plasma membrane is rare. Occasionally a few mitochondria were observed in the cell periphery. Internal to the granular layer there occurs a longitudinal layer of fine filaments which show no internal structure (figs. 1, 3, and 4). The perinuclear portion of the cytoplasm contains the mitochondria, Golgi complex, endoplasmic reticulum, and lipide bodies (figs. 3 and 4). The mitochondria are relatively small and cylindrical, averaging 290 by 560 millimicrons. They contain few cristae and possess a matrix of relatively low density. The Golgi complex consists of few parallel lamellated saccules and many vesicles (figs. 1 and 6). It is generally situated very close to the nucleus. In one cell the Golgi complex comprised only vesicles whose membranes were relatively thick and whose internum was dense (fig. 7). The endoplasmic reticulum consists generally of few scattered vesicles whose limiting membranes have both smooth and rough profiles (figs. 3 and 5). Lipide bodies are numerous and generally associated with the endoplasmic reticulum and mitochondria in the perinuclear cytoplasm.

The nucleus is usually centrally situated and of smooth outline. The perinuclear cisterna is variable in width and numerous pores may be present. The nuclear matrix is composed of granules of varying sizes and densities. The nucleolus occupies a relatively large portion of the nucleus.

In several instances necrotic cells were observed. Such cells (fig. 8) show small dense particles in the cytoplasm. These particles differ from those observed in the transformed amnion cells in that they possess a homogeneous internal structure and are not bounded by membranes.

b. Cultures of Transformed Amnion Cells (Strain FL)

The ultrastructure of the FL cells is strikingly different from that of the primary amnion cells. Further, the differences between cells from young cultures (3 days after seeding) and cells from old cultures (6–7 days after seeding) are such that they will be considered separately.

Three-day-old cultures.—The cell border consists of plasma membrane only and is remarkably uniform in outline. The cytoplasm has a general "empty" appearance, the matrix consisting of dispersed granules. One of the outstanding characteristics of the cells from the young cultures is the structure of the numerous mitochondria (figs. 9 and 10). These are generally cylindrical and long, and many are branched in a manner suggestive of budding. They possess few cristae and have a relatively low-density matrix. The mitochrondria average 0.37μ in diameter and may be up to 5.10μ in length. The cristae are often incomplete and may be parallel, branched, or recurrent.

The endoplasmic reticulum is abundant, occurring in the form of vesicles and tubules, with the vesicular component predominating. The membranes of the reticulum are generally smooth, but some reticular tubules with attached Palade granules are observed. The interior of the reticulum is generally empty, but occasional reticular vesicles contain a relatively dense substance (fig. 11). The Golgi complex varies from a small organelle close to the nucleus (fig. 12) up to a widely scattered system of lamellae and vesicles occupying a large portion of the cytoplasmic area (fig. 13).

The most notable feature of the cells from the 3-day-old cultures is the presence of numerous spherical and membrane-limited structures (figs. 13 through 18) herein designated as multivesicular bodies. These may occur throughout the cytoplasm but are most numerous in and near regions filled with vesicles and membranes of the Golgi complex (figs. 13 through 17). The multivesicular bodies vary little in size in the plane of section, averaging 400 to 570 mμ in diameter. The body consists of a single membrane enclosing about 20 to 50 small vesicles whose diameters range up to 38 mμ, with the majority 24 to 38 mμ in the plane of section. The small vesicles usually consist of a single membrane, but they may enclose a second particulate component of variable density. The outer membrane of the multivesicular bodies, in the periphery of the cytoplasm, may be continuous with the plasma membrane of the cell or with vesicles continuous with the plasma membrane (fig. 18). In the Golgi zone the

incomplete membrane of the multivesicular body appears to be closely associated with Golgi membranes (fig. 16).

The variety of forms assumed by the multivesicular bodies presumably reflects stages in their growth. Some comprise a single membrane enclosing small vesicles of almost uniform density and size (fig. 16). Some have an incomplete enclosing membrane (figs. 16 and 18). In many, one or more dense, eccentric, vesicular aggregates occur ("multivesicular nucleoids"—*cf.* Sotelo and Porter, 1959) (figs. 15, 16, and 17). In some cells the multivesicular bodies appear to contain closely packed vesicles and particles of considerable density limited by either a complete membrane (figs. 13 and 15), or incomplete membrane (fig. 17). Small vesicles of a size and density similar to those within the multivesicular bodies are also observed free in the cytoplasm. These most frequently occur near the lamellae of the Golgi complex (figs. 12 and 14), but also may be aggregated around the limiting membranes of the multivesicular bodies (figs. 15 and 16). They are probably closely related to the small vesicles within the enclosed multivesicular bodies, having been either discharged from a disrupting multivesicular body or, in the process of aggregating, enclosed by the membrane of a newly forming body.

With less frequency, concentric vesicles and dense large particles (fig. 19) have been observed. The latter have a size similar to that of the dense particles common in cells of older cultures, but lack a resolvable internal structure. Irregular formations of lipide may be scattered throughout the cytoplasm (fig. 10), apparently not closely related to a specific membrane system.

The nucleus is large, with relatively many pores, and its outer membrane often appears continuous with membranes of the endoplasmic reticulum and, directly or indirectly, with the outer membrane of adjacent mitochondria.

Six- and 7-day-old cultures.—The cell border is highly variable, smooth, or with rounded or streamer-like projections (fig. 20). The projections contain little cytoplasm and often have small vesicles. In the culture the cells are fairly tightly packed, usually by plasma-membrane apposition (figs. 23, 25, and 26). In rare instances intercellular bars (desmosomes) have been observed. These appear as local areas of dense material associated with the thickened plasma membrane (fig. 21) or as parallel membranes facing each other in adjacent cells.

Characteristically, the cytoplasm is filled with granules, fine filaments, vesicles and tubules of the endoplasmic reticulum, mitochondria, and lipide (fig. 22). Relatively empty areas have been observed in only a few cells. In such areas the matrix consists of extremely fine filaments, closely packed, surrounded by Palade granules, and tubules and vesicles of the endoplasmic reticulum (fig. 23). The initial appearance of the fine filaments in regions of concentrated and aligned Palade granules and ergastoplasmic tubules suggests that the latter are active in the filament formation. The mitochondria are numerous, scattered generally throughout the cell, and most commonly, roughly spherical. They may be rela-

tively empty with few incomplete cristae or they may have many complete parallel cristae and a dense matrix. The endoplasmic reticulum appears in various cells in two distinctly different forms. In those cells showing filamentous areas the rough profiles of the reticulum are arranged in parallel concentric layers (fig. 23) surrounding the filamentous area. In most cells the membranes of the reticulum appear as smooth membraned vesicles (figs. 22 and 24) which show no preferred site within the cell.

The Golgi complex is a prominent feature of these cells. It has three components: microvesicles, lamellated saccules, and macrovesicles. In some cells the Golgi complex is relatively small and situated near the nucleus (fig. 24). More commonly the membranes of the Golgi complex occupy large areas of the cell (fig. 25), the microvesicular component in such areas being the most common. Closely associated with the Golgi area are multivesicular bodies resembling those observed in the young cultures.

The most striking structures in the cells from the older cultures are the membrane-limited particles of complex inner structure (figs. 26 through 34). There appear to be several types: (*a*) Particles, enclosed within one or more membranes, occur with some frequency in the peripheral cytoplasm (figs. 27 through 30, and 34). They consist of an outer dense membrane, a less dense area, a second dense membrane, and a central area of medium density containing one or more dense membranous particles. The outer membrane averages 100 to 180 mμ in diameter, the inner membrane averages 50 to 100 mμ, and the contained single-membraned, dense particles average 25 to 30 mμ in diameter in the planes of sections. In some examples the contained dense membranous particles may be eccentric, but in general they occupy the center of the medium dense area. (*b*) Large spherical membranous structures (170–200 mμ) occur, with up to 6 concentric layers of membranes (figs. 26, 27, 32, and 36). The density of these membranes is greater than that of the vesicles of the endoplasmic reticulum. (*c*) Granular, round-to-ovoid, dense bodies of a similar diameter (figs. 26, 27, and 32) occur in the same areas. (*d*) Small (30–45 mμ) vesicles, both free and contained within multivesicular bodies (figs. 26, 31, 35, and 36), may also be present in the peripheral cytoplasm.

The large and small particles not only occur in the interior of the cell but also may be seen in the peripheral cytoplasm just beneath the cell border (fig. 27), or even free in the intercellular space (fig. 33).

DISCUSSION

Comparison of cells in the normal human amniotic membrane after trypsinization in primary tissue cultures, and after transformation in continuous culture, reveals that each stage has a recognizably characteristic cell architecture. While still in the amniotic membrane, the indi-

vidual cells have microvilli, few mitochondria, few intracellular membranes, and basement membrane material on the stromal border, and they are closely apposed by intercellular bars (desmosomes). After trypsinization these cells lose their bar attachments and basement membrane material, and show a hypertrophy of endomembrane systems (endoplasmic reticulum, Golgi complex, and mitochondria), which are changes possibly due to the lack of regulatory influence by the organismic milieu and to the mobilization of intracellular resources in preparation for survival as unicellular organisms (*7*).

Once established in tissue culture the cell no longer resembles either original amnion cell or trypsinized cell in that it has organized layers of organelles and bands of fine filaments in the cytoplasmic matrix. The primary cultured cells appear to be trypsinized cells with a decreased cytoplasmic mobility resulting from altered relations among the endomembranes responsible for the synthesis and organization of linked enzyme systems.

Biologically, the fast-dividing, hypertriploid FL cells, capable of producing tumors characterized as malignant growths in conditioned animals and in human volunteers, differ morphologically from the previous stages, and vary among each other depending on culture age. The cells of young cultures have uniform borders, "empty" cytoplasm, large mitochondria, and abundant ergastoplasm. Further, these cells have an extremely proliferated Golgi complex and related multivesicular bodies. Surprisingly enough, when the FL cell culture is twice as old, the cells differ radically from those of the younger subculture. Their borders are variable, with cytoplasm full, mitochondria small, reticulum unorganized, and Golgi complex large and dispersed. The multivesicular bodies are seen only rarely in the older cultures. However, the isolated complex particles and vesicles seldom observed in the previous stage are common in the older cultures.

Most ultrastructural changes observed can be related to changes in environment and biologic behavior. The loss of villi and basement-membrane material is associated with the change from specialized function in the fixed tissue position to the nonspecialized free unicellular life in trypsin or the loose cellular apposition in the tissue-culture monolayer. The appearance of intercellular bars in the few instances observed in an older FL culture suggests that its cells are forming a more solid, fixed tissue, in which intercellular influence is important. The size, number, and distribution of mitochondria appear directly related to the oxidative metabolism involved in synthesis and growth. In the trypsinized cell one can interpret the hypertrophy of the mitochondria as being related to the energy production necessary for membrane synthesis. In the primary cultures the small number of mitochondria, plus reticulum, seem related to low-growth rate. The long, often branching mitochondria of the 3-day FL cultures indicate rapidly growing cells, and are reminiscent of the types seen in fast-growing embryonic and newborn heart cells (*8*), and in brown adipose tissue of a newborn mouse (*9*). The cells of the older

cultures presumably have less oxidative activity as indicated by the scarce, relatively small, and dense mitochondria. The RNA granules alone, and together with the endoplasmic reticulum (ergastoplasm), are undoubtedly associated with synthesis of protein (*10*), whether it is membrane replication in the trypsinized cells and FL cells of the younger cultures, or filament formation in the primary cultured cells.

Of particular interest in cell transformation is the Golgi complex. Exaggerated development and dispersion of the Golgi complex was noted earlier in the trypsinized normal cells (*7*). A similar profusion of Golgi membranes may occur in neoplastic cells with predominance of the microvesicular element (*11–14*). That the Golgi complex may be involved in the formation of particles was first suggested by Bernhard, Guerin, and Oberling (*15*). Further evidence has been brought forth for a mouse-plasma cell tumor (*16*), and it has been observed that "virus-like" particles in mouse-mammary tumor may be initially associated with the Golgi complex and that later the inclusions occupy the areas previously occupied by the Golgi zone (*17*). It is thus particularly significant in the present study that the multivesicular bodies of the FL cells from 3-day cultures and the particles of the 6- to 7-day cultures are intimately related to the Golgi complex, and in some instances the membranes for the intracytoplasmic membrane-limited particles appear to be continuous with Golgi membranes. The Golgi complex appears to be an excretory organelle, herein specifically acting in the formation transport and elimination of microvesicular-contained material.

Noteworthy in the transformed and malignant FL cells is the presence of multivesicular bodies and the membrane-limited intracytoplasmic particles. The isolated, larger, membrane-limited particles predominating in the cells of older cultures resemble, in some of their morphologic features, the virus-like particles of certain animal tumors (*1*, *15*, *17–25*). Variations in particle density and membrane relations do occur. Whether or not directly pertinent to the present study, it should be noted that morphologic variations in particles have been observed in mouse tumors (*15*, *17*), and well illustrated for all tumors, in a recent review (*11*).

The presence of the variety of forms of multivesicular bodies and their relation to the Golgi complex and to the free, small, uniform vesicles in younger cultures suggest their relation to growth stages of the transformed cells. The relation of the multivesicular bodies to the malignancy of the FL cells can only be speculated on. At the least, two possibilities exist. Multivesicular bodies, or small vesicles within and around membrane-limited vacuoles of similar size, have been observed in monkey kidney cells infected with herpes and poliovirus and in eosinophils from an infected mouse spleen (unpublished observations). Similar structures in mouse mammary carcinoma have been observed (*25*) and the possibility suggested that they represent a cellular reaction to injury or infection and do not participate in the formation of membrane-limited particles. Recently (*26*) multivesicular bodies in a number of normal cells, particularly the rat ovum, have been described and the suggestion made that

these are self-reproducing, vesicle-forming constant components of animal cells related to the cellular growth cycle. Thus one can assume that either (*a*) these bodies are cellular reactions to the factor or factors responsible for the malignancy of the FL cells, or (*b*) they are normal components of the rapidly growing FL cells and evidence of the rapidity of growth. In either event their function remains to be determined.

The possibility cannot be excluded that the membrane-limited particles could be noninfectious structures related to culture growth, or could be extraneous, noncytopathogenic viruses that accidently infected the cultures and were carried from passage to passage, appearing under certain optimal conditions present in the cultures studied. All the evidence given, however, strongly suggests that these particles are intimately related to the transformation of normal human amnion cells into malignant cells. Morphologic and biologic studies of cell fractions are now underway to determine whether the observed suspected particles are indeed infectious.

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PLATES

PLATE 76

Human amnion cells in primary culture

FIGURE 1.—Cell border with large irregular projections (pr). The filamentous layer (f) is peripheral; mitochondria (mi) and Golgi complex (g) central. × 30,000

FIGURE 2.—Cell border sometimes shows thin, almost microvillous, projections (pr). Smooth membrane-bound vesicles of endoplasmic reticulum (er) scarce. Nucleus (nu) usually ovoid and smooth in outline. × 30,000

FIGURE 3.—Interior of cell showing nucleus (nu) with wide perinuclear cisterna. Perinuclear cytoplasm contains 2 lipide bodies (L), rough membrane-bound profiles of endoplasmic reticulum (er), small mitochondria (mi), and fine filaments (f). × 30,000

FIGURE 4.—Cell with smooth border and peripheral filaments (f). Small mitochondria (mi) near smooth-outlined nucleus (nu). × 30,000

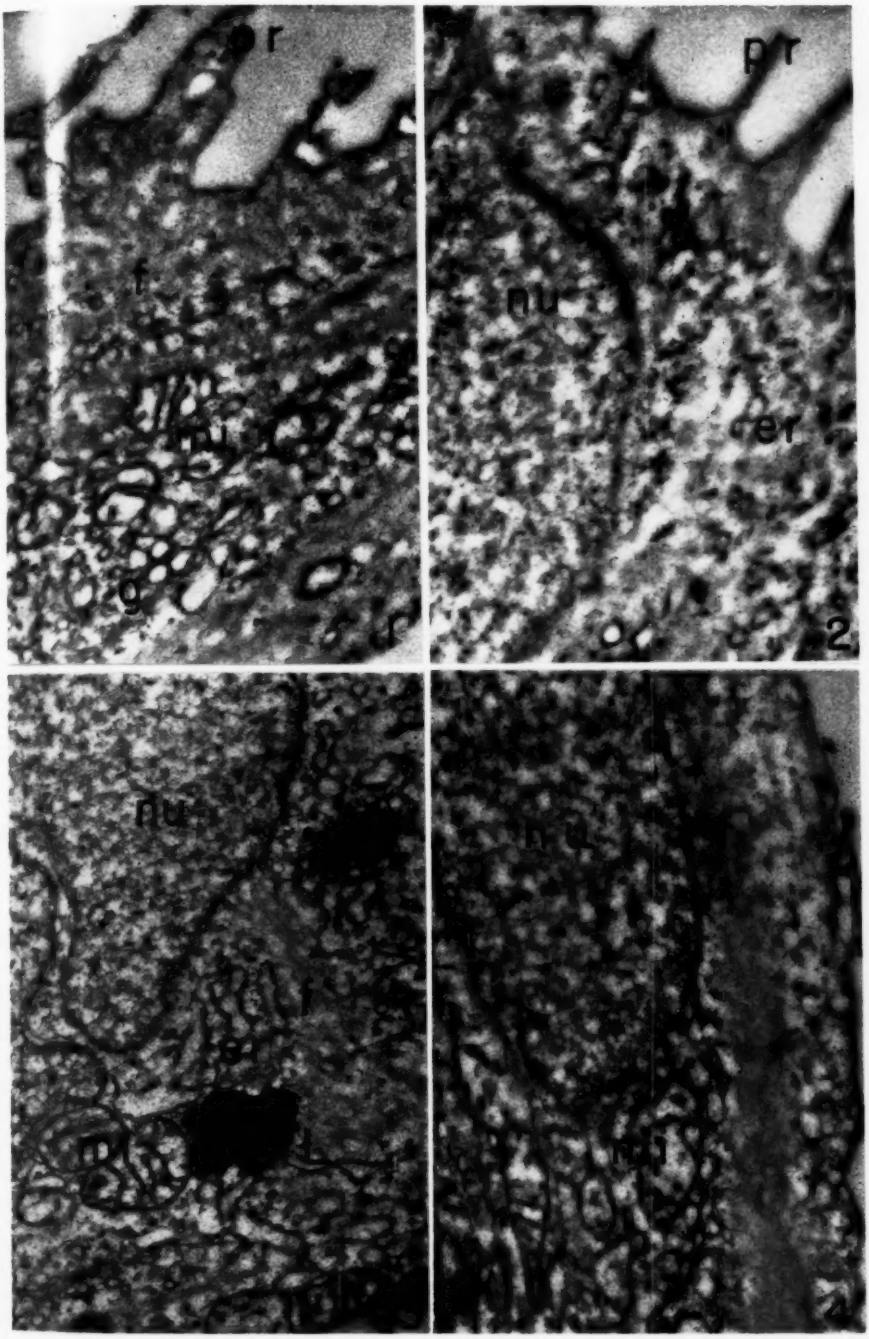

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PLATE 77

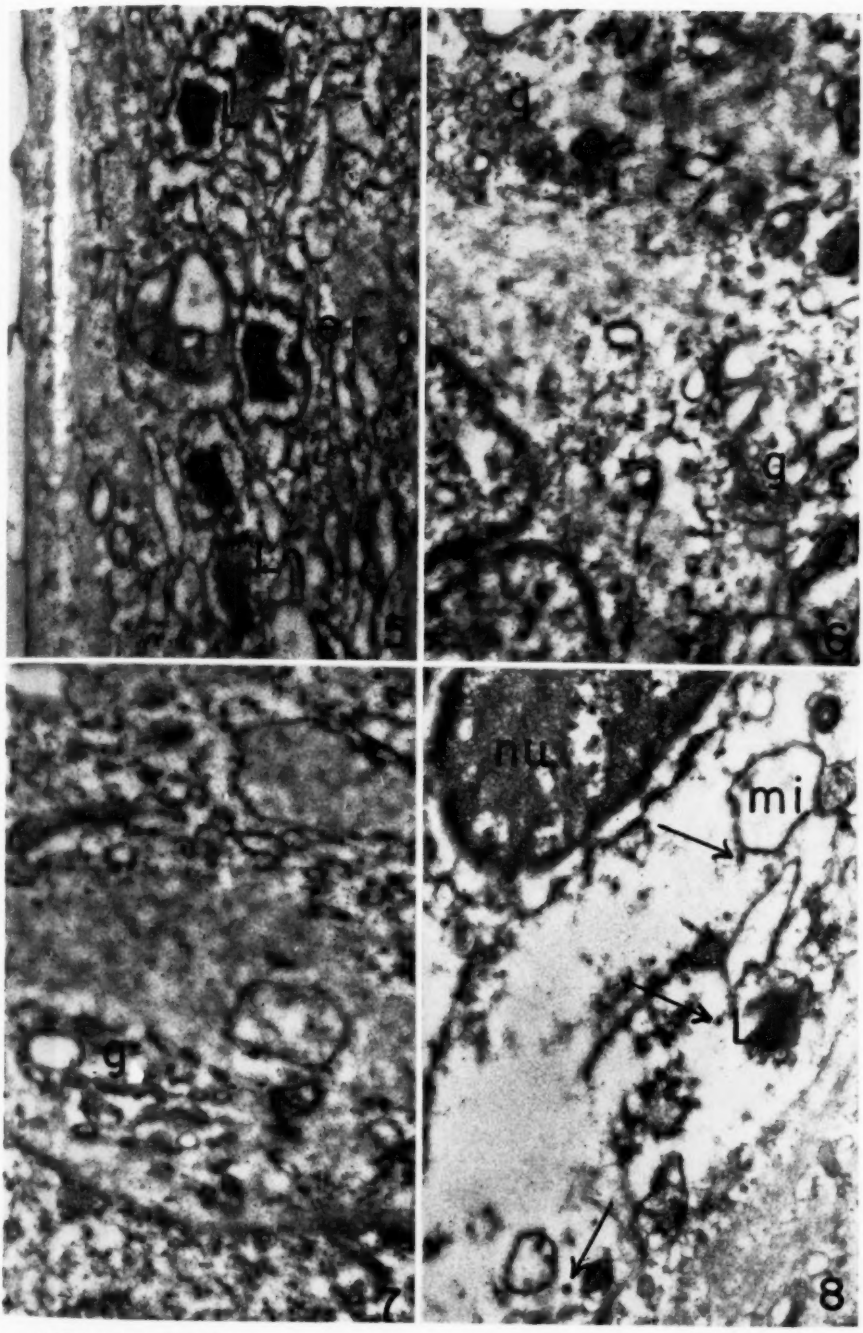
Human amnion cells in primary culture

FIGURE 5.—Cell containing many lipide deposits (L) s. nded by membranes of the endoplasmic reticulum (er). Peripheral, filamentous cytoplasmic layer, and smooth cell border at *left* of micrograph. × 30,000

FIGURE 6.—Rarely in primary cells is the Golgi complex (g) disperse as shown here. More often it comprises a small aggregate of lamellae and vesicles near the nucleus. × 30,000

FIGURE 7.—An exceptional Golgi complex (g) in which the vesicular components are thick-walled and contain a dense substance. × 30,000

FIGURE 8.—Necrotic cell with fragments of nucleus (nu), lipide droplets (L), mitochondrial membranes (mi), and few small dense particles (*arrows*). × 30,000



FOGH AND EDWARDS

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PLATE 78

FL cells from 3-day cultures

FIGURE 9.—Long, relatively empty mitochondria (mi) often show branches in which the cristae run normal to the mitochondrial body. × 25,000

FIGURE 10.—Often nonmembrane-limited lipide deposits (L) are seen in close association with the long mitochondria (mi). × 25,000

FIGURE 11.—In young culture cells the tubules of the endoplasmic reticulum (er) may contain a dense substance. The cytoplasmic matrix contains a large number of RNA granules. Near the mitochondrion (mi) at bottom of micrograph may be seen a multivesicular body (mvb) and an adjacent membrane-limited particle (*arrow*). × 40,000

FIGURE 12.—Cytoplasm in proximity of nucleus (nu). The Golgi complex (g) predominantly microvesicular. Dense vesicles and particles (*arrows*) similar to those contained within the multivesicular bodies often occur in Golgi zone. × 33,000

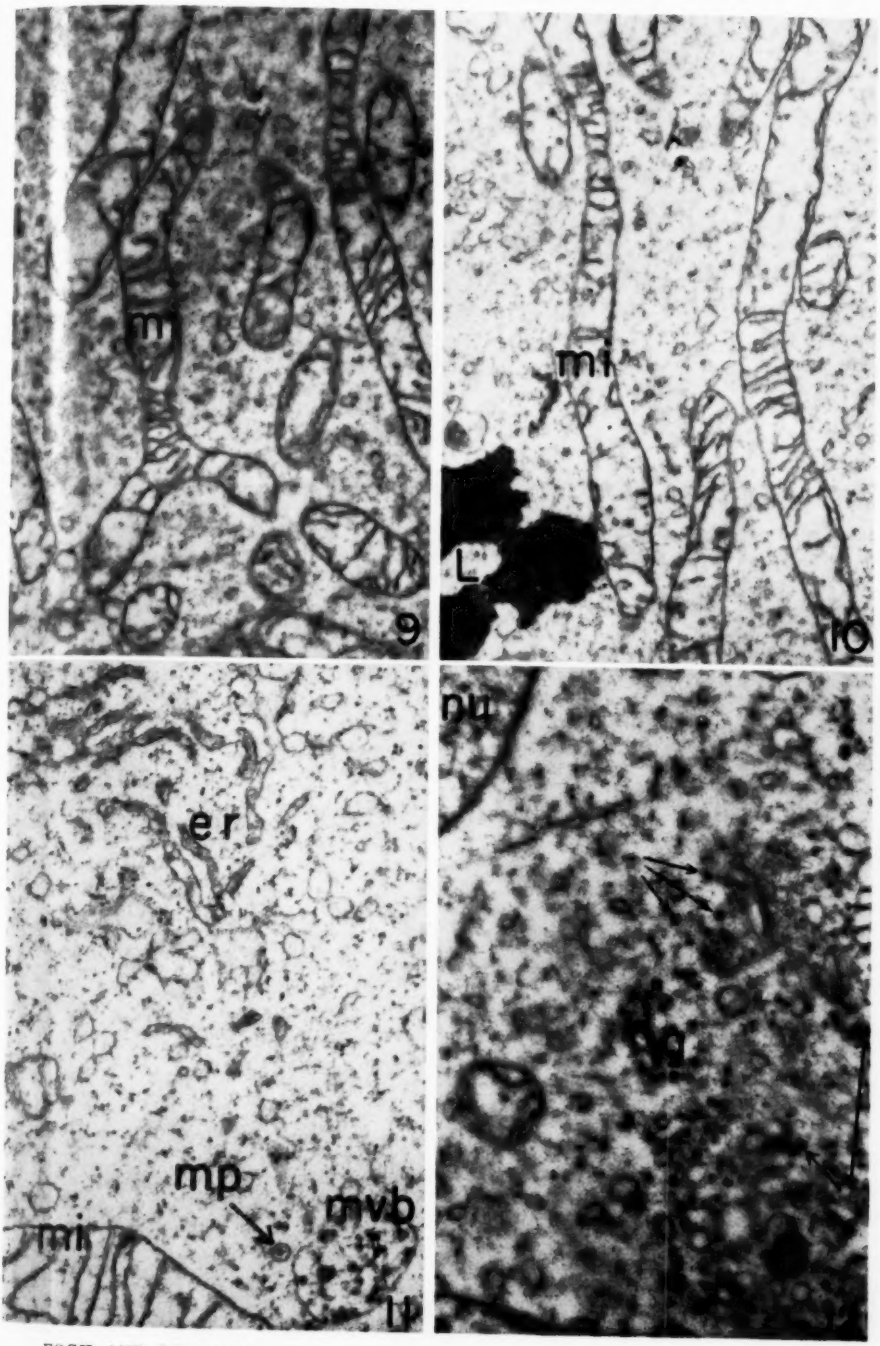

m
9
mi
L
10
er
mp
mvb
mi
11
pu

PLATE 79

FL cells from 3-day cultures

FIGURE 13.—Excessively developed Golgi zone (g) throughout which may be seen free, uniform, small, dense vesicles, single membrane-limited multivesicular bodies (mvb), and double membraned mitochondria (mi). × 30,000

FIGURE 14.—Multivesicular bodies (mvb) in Golgi zone (g). *Note* that the membrane of the 2 lower bodies is incomplete. Nucleoid (n) of multivesicular body is an eccentric dense aggregate of contained vesicles. × 45,000

FIGURE 15.—At *upper right* double membrane-limited particle (mp). Also at *upper right* a multivesicular body (mvb_1) with tightly packed dense vesicles. Nucleoids (n) appear in 2 of the multivesicular bodies (mvb_2, mvb_3). Lower multivesicular body (mvb_4) has incomplete membranes. × 50,000

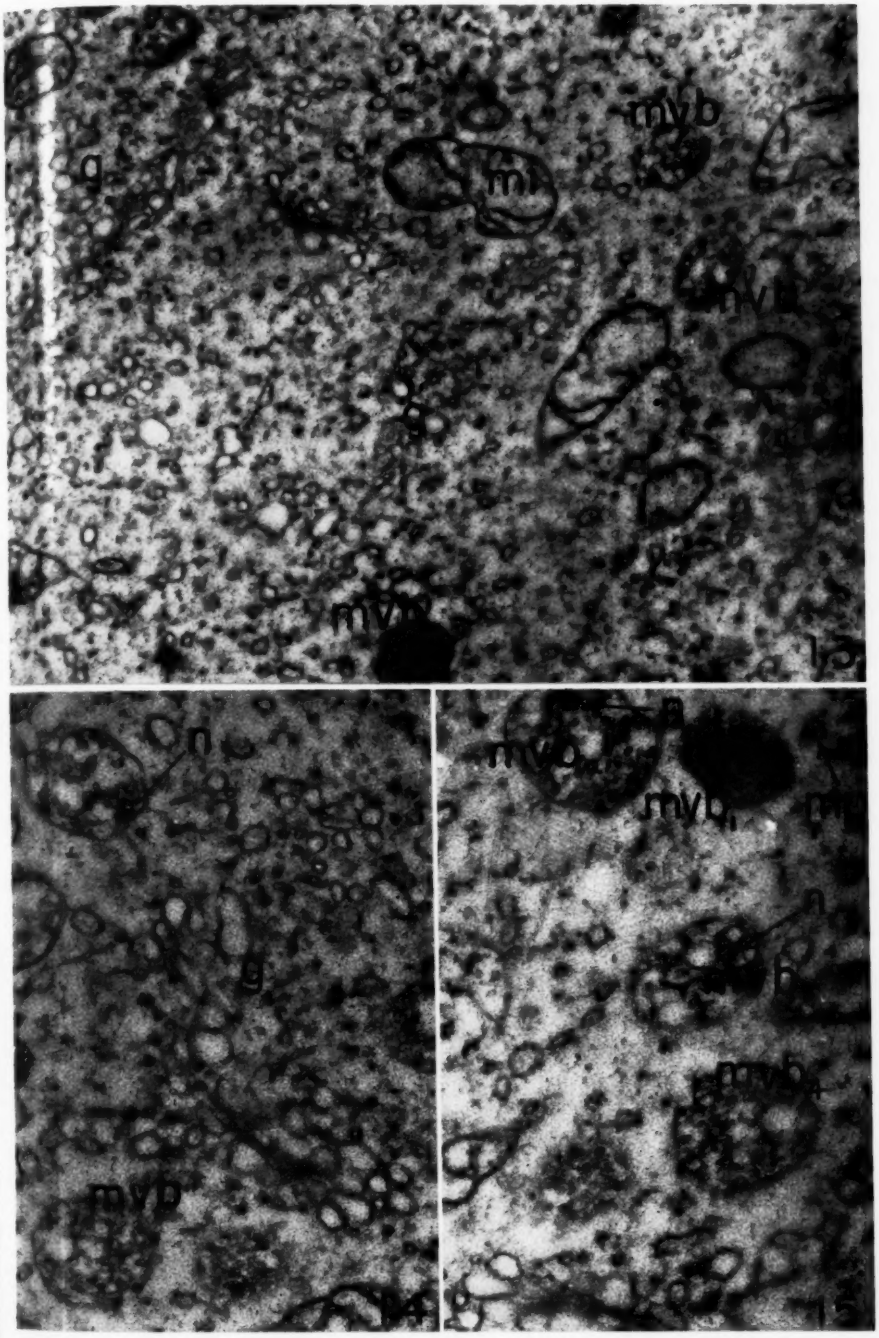

mvb
g
mi
mvb
mvb
mvb
g
mvb
mvb
mvb
g
mvb

PLATE 80

FL cells from 3-day cultures

FIGURE 16.—Golgi zone (g) of cell and associated multivesicular bodies (mvb); some containing nucleoids (n), and others both light and dense vesicles; some enclosing membranes complete (mvb_1) and some incomplete (mvb_2) suggestive of either a secondary membrane formation around aggregating vesicles or disruption of the multivesicular body with consequent freeing of vesicles. × 36,000

FIGURE 17.—Golgi zone (g) with dense forms of multivesicular bodies (mvb), some of which contain nucleoids (n). × 50,000

FIGURE 18.—Mitochondria (mi) and multivesicular bodies (mvb) in cell periphery. Of particular interest are the 2 bodies at cell border whose enclosing membranes are continuous with the plasma membrane of the cell (*opposite arrows*). × 50,000

FIGURE 19.—In some cases several different types of particles and vesicles are seen in 3-day cultures. Micrograph shows portion of nucleus (nu) at *left*, scattered "empty" mitochondria (mi), small dense vesicles (v), concentric vesicles (cm), and large dense membrane-limited particle (dp). × 40,000

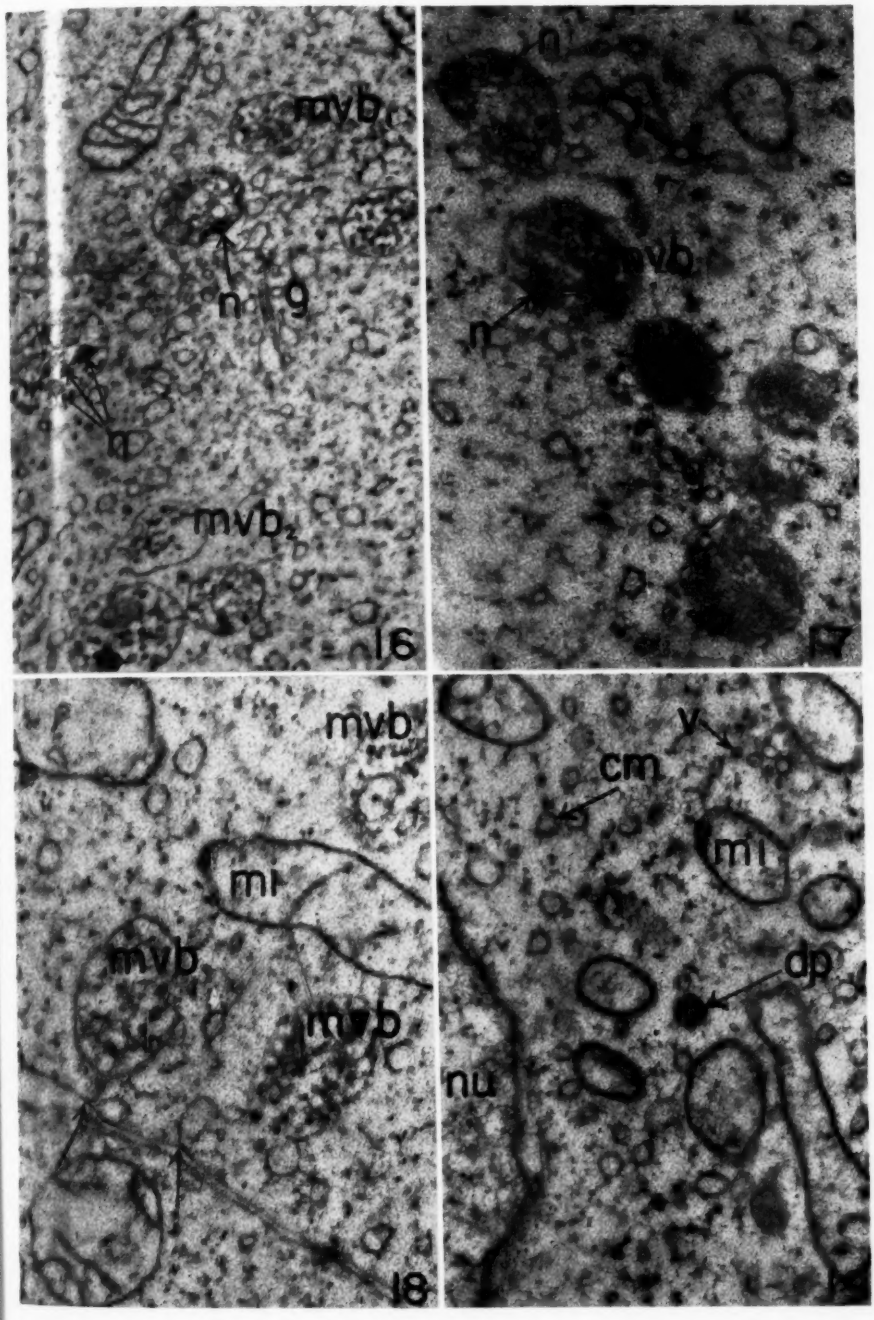

mvb
n
g
mvb
16
n
mvb
17
mvb
mi
mvb
mvb
18
v
cm
mi
dp
nu

PLATE 81

FL cells from 6- and 7-day cultures

FIGURE 20.—Although the cells at this stage are more tightly packed than previously, the cell borders often consist of long irregular projections. × 30,000

FIGURE 21.—In only 3 of hundreds of preparations were indications seen of intercellular influence similar to intercellular bars (desmosomes). These were plasma membrane thickenings (*arrows*) and multiple layers of membranes at the cell surface. × 40,000

FIGURE 22.—Characteristically the cytoplasm contains an abundance of RNA granules, relatively small mitochondria (mi), smooth membrane-limited profiles of endoplasmic reticulum (er), lipide droplets, disperse Golgi complex (g), and particles of various sizes. × 30,000

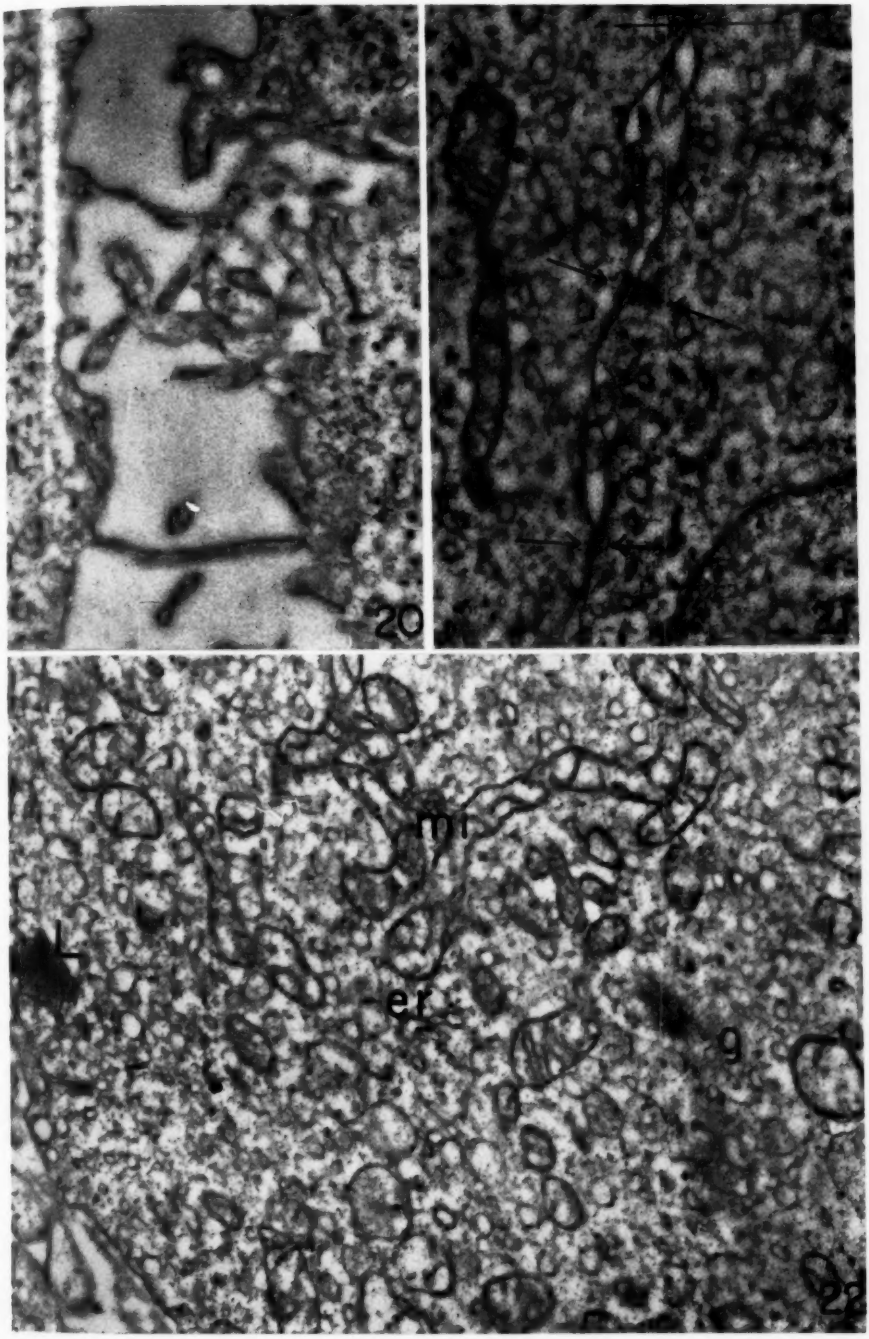

20
mi
er
9

PLATE 82

FL cells from 6- and 7-day cultures

FIGURE 23.—Concentric layers of rough membrane-limited tubules of endoplasmic reticulum (er) were seen in those few cells whose cytoplasmic matrix was filamentous (f). × 30,000

FIGURE 24.—Endoplasmic reticulum (er) more commonly was composed of smooth membranes. In this instance the Golgi complex (g) is small and near nucleus (nu). × 30,000

FIGURE 25.—More often the Golgi complex (g) is widely dispersed throughout the cell. × 30,000

FIGURE 26.—Peripheral cytoplasm of 2 cells showing free vesicles (v), particles, multivesicular bodies (mvb), large bodies of concentric membranes (cm), and numerous membrane-limited granular bodies (gb) of medium density. × 35,000

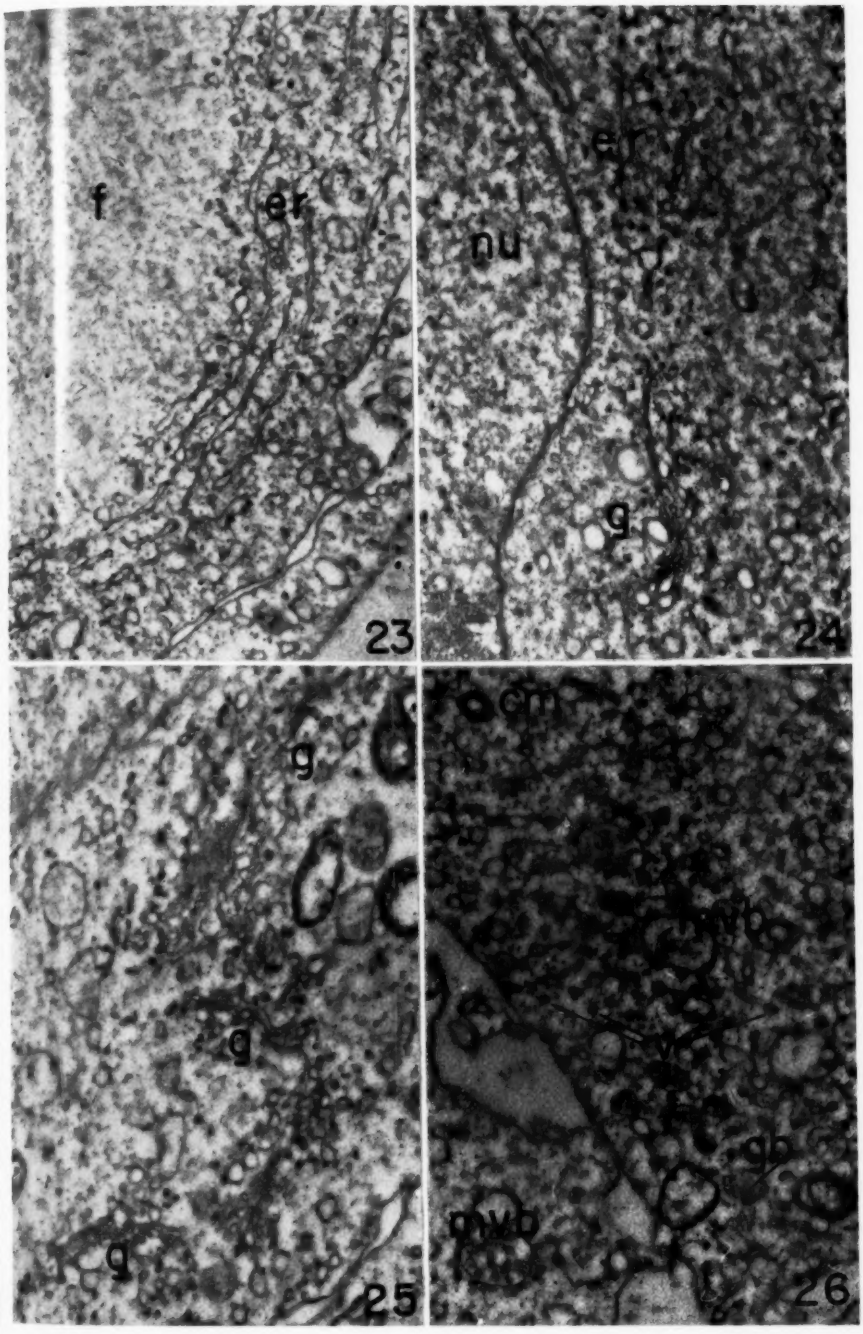

f
er
23
er
nu
g
24
g
g
g
25
ocm
mvb
gb
mvb
26

PLATE 83

FIGURE 27.—Vesicle- and particle-filled cytoplasmic area of cell from 7-day culture. Predominating are small dense vesicles and intermediate-sized particles (dp) limited by either 1 or 2 membranes. At *upper right* is a dense body composed of concentric membranes (cm) to the *left* of which may be seen several bodies (gb) of a similar size, granular, and of medium density. × 60,000

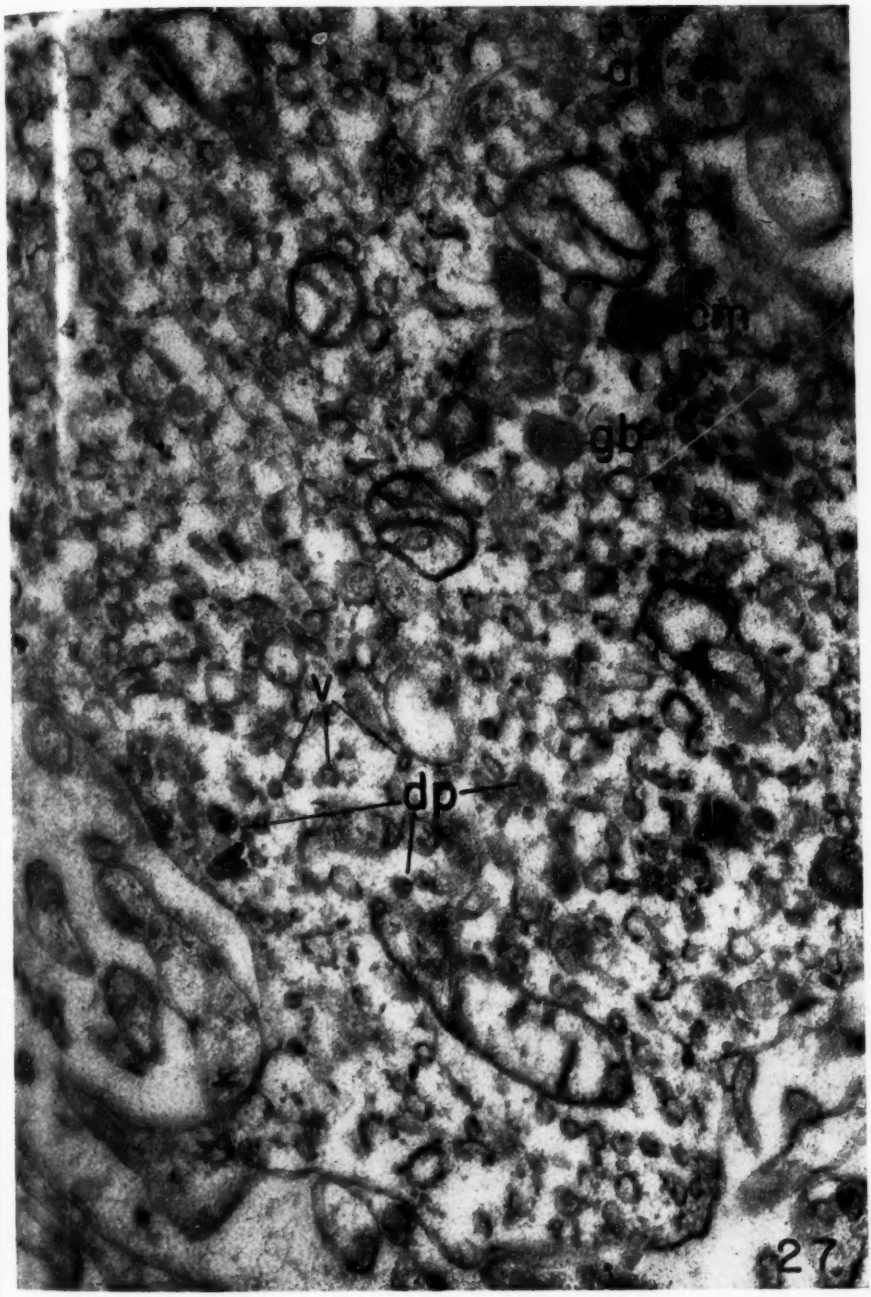
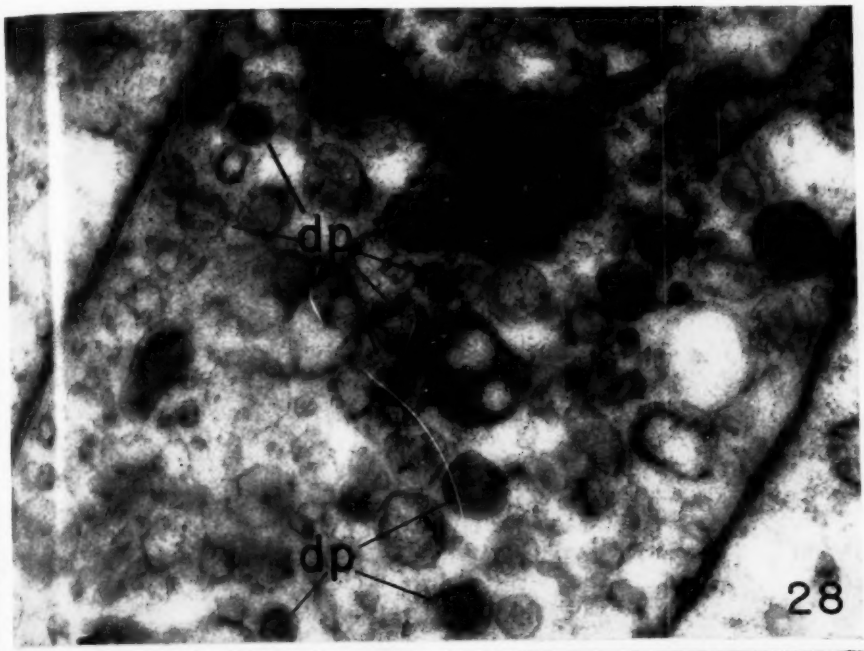

cm
gb
v
dp
27

PLATE 84

Particle-containing areas of FL cells from 6- and 7-day cultures

FIGURES 28 AND 29.—Populations of dense particles (dp) bounded generally by 2 membranes. The core of the particles consists of smaller, dense, membranous particles of 25 to 30 mμ diameter. × 60,000


dp
dp
28

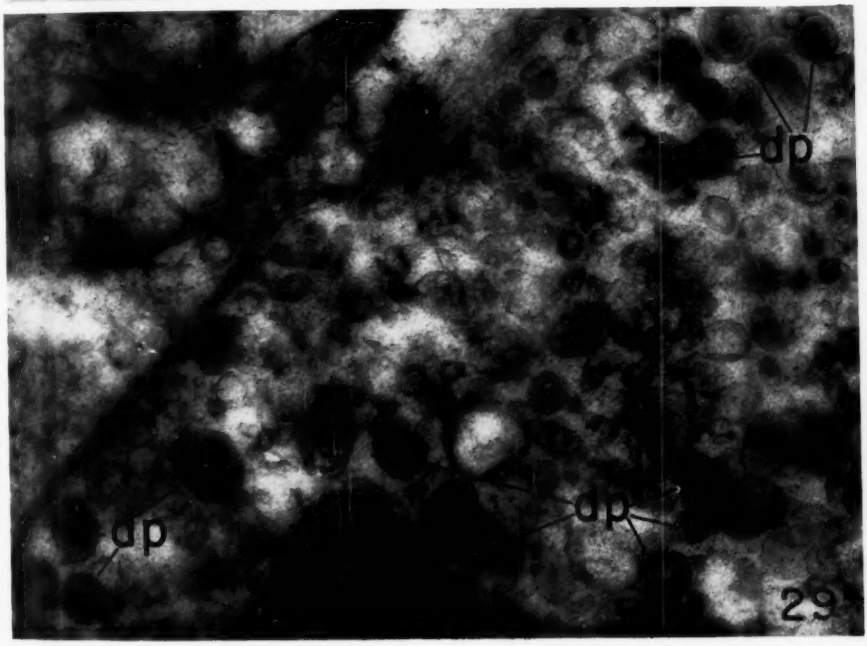

dp
dp
dp
29

PLATE 85

Particle and vesicle-containing areas in FL cells from 6- and 7-day cultures

FIGURE 30.—Double membrane-bound particle (p) in peripheral cytoplasm. × 50,000

FIGURE 31.—Small dense vesicles aggregated in vesicular bodies (mvb) and free (v) in cytoplasm. × 35,000

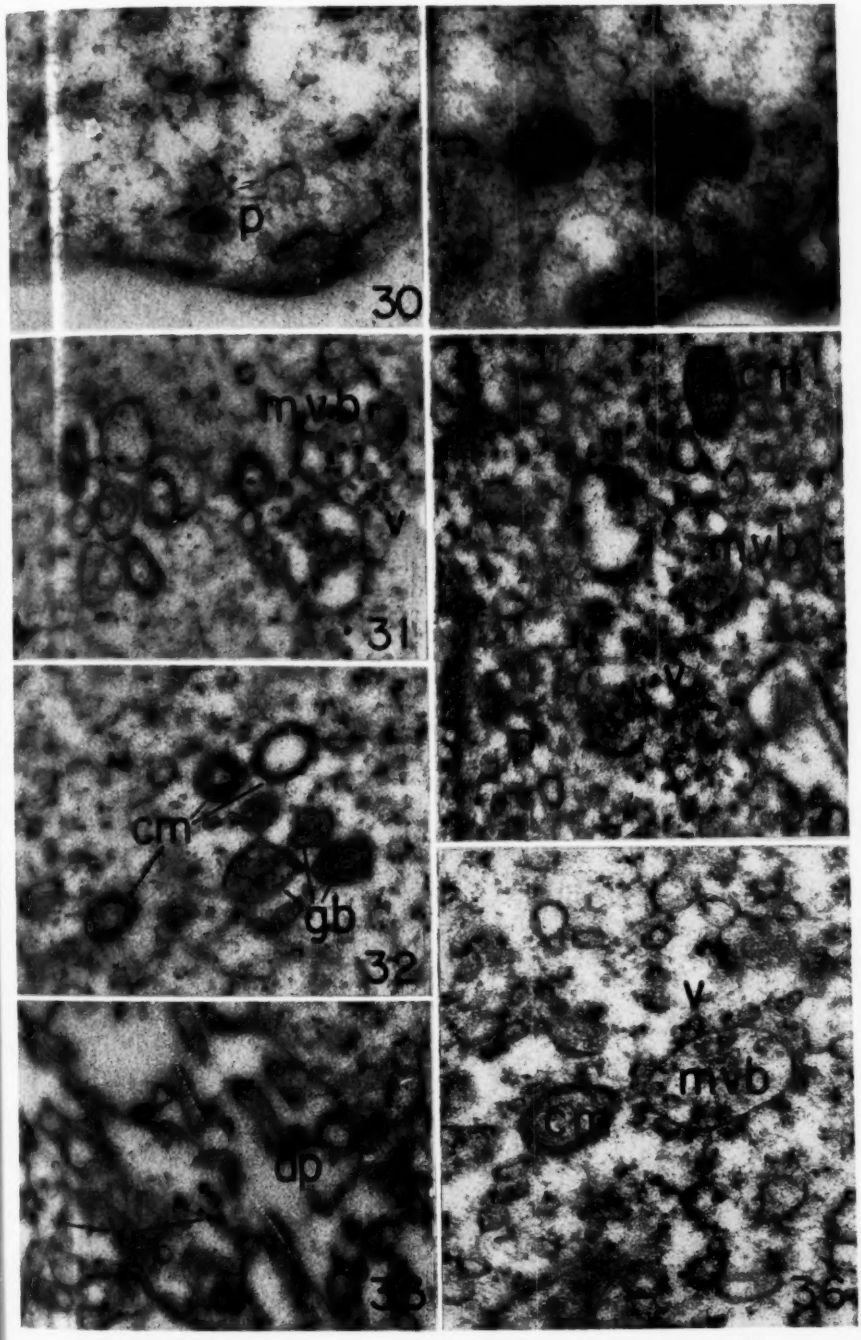
FIGURE 32.—Bodies comprising concentric layers of membranes (cm) and granular bodies (gb). × 50,000

FIGURE 33.—Small vesicles (v) in cytoplasm and dense particles (dp) in intercellular space. × 30,000

FIGURE 34.—Two dense and several less dense, double membrane-limited particles. × 60,000

FIGURE 35.—Dense, concentric-membraned body (cm), multivesicular bodies (mvb) with discontinuous membranes, free vesicles (v) in adjacent cytoplasm, and membrane-enclosed particle (p). × 60,000

FIGURE 36.—Concentric-membraned body (cm) and multivesicular body (mvb) showing contained vesicles aligned at periphery and free vesicles (v) in the cytoplasm aligned immediately around membrane of multivesicular body. × 60,000


p
30
m.v.b
v
31
cm
m.v.b
v
cm
gb
32
dp
33
v
m.v.b
cm
36

Mechanism of Experimental Tumorigenesis. I. Epidermal Hyperplasia in Mouse Caused by Locally Applied Tumor Initiator and Dipole-Type Tumor Promotor [1, 2]

KAI SETÄLÄ, LAURI MERENMIES, LEO STJERNVALL, YRJÖ AHO,[3] *and* PAAVO KAJANNE,[3] *Department of Pathology, University of Helsinki, and Department of Chemistry, Institute of Technology, Helsinki, Finland*

SUMMARY

Laboratory-synthesized "membrane active agents," Span 60 (sorbitan monostearate) and its ethylene oxide derivative, Tween 60 (polyoxyethylene sorbitan monostearate), and Tween 20 (polyoxyethylene sorbitan monolaurate), produced changes in skin on back of the mouse analogous to those caused by corresponding technical products. The degree of hyperplasia was correlated to the tumor-promoting property of the agent, or to its absence. The skin changes produced by a potent tumor promoter of Tween 60 type, which were analyzed histologically and histoquantitatively, differed from those caused by similarly applied carcinogens methylcholanthrene (MCA) and 9,10-dimethyl-1,2-benzanthracene (DMBA). A single application with MCA or DMBA immediately produced toxic alterations. Reparative hyperplasia set in only after the latter became quiescent. Continuous MCA or DMBA treatment caused cellular and nuclear atypia and severe disturbances in organization and differentiation, which increased progressively. Treatment with Tween 60 did not produce retrogressive alterations, but intense cell multiplication and high-degree hyperplasia commenced immediately. Continuous Tween 60 treatment caused neither cellular nor nuclear atypia, nor disturbances in organization; the amount of cells in differentiation increased significantly. Continuous treatment maintained the hyperplasia unchanged. After initial application of carcinogen, Tween 60 treatment evoked changes typical of carcinogens. Tween 20 (a weak tumor promoter) and Span 60 (an ineffectual compound) did not produce these alterations. The nature of tumor promotion after application of a pure dipole-type promoter is benign. Tumor promotion is not a phase of the carcinogenic process itself as these processes are contradictory. Tumor promotion is comparable to intense continuous reparative ("simple") cell multiplication.—J. Nat. Cancer Inst. 23: 925–951, 1959.

SINCE 1945 we have used certain "membrane active agents" in studying the mechanism of tumorigenesis (*1*). Technical dipole-type tumor promoters produce a high degree of epidermal hyperplasia in the mouse (*2–5*).

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[3] Department of Chemistry, Institute of Technology, Helsinki, Finland.

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The degree of hyperplasia is always positively correlated to the concentration and tumor-promoting property of the compound used (*2*, *5*). When treatment with these substances is continuous, the hyperplasia remains unchanged for as long as 60 weeks (*4*). The tumor incidence depends on the frequency of application: The oftener an aqueous compound (in aqueous state) is applied to the skin, the greater the number of tumors (*5*, *6*). Light and electron microscopic observations (*7–9*) gave consistent evidence that tumor promotion in the mouse skin should be regarded as comparable to benign, reparative cellular proliferation. This is corroborated by the classical discovery of Deelman's (*10*), in which the proliferation that occurs in wound healing promotes the development of skin tumors [*cf.* Mottram (*11*)].

On the basis of our previous studies we feel convinced that tumor promotion, under the experimental conditions used, is not carcinogenic. Further supporting evidence is the observation made in this laboratory that a large number of pure fatty acids, which form the lipophilic portion of the membrane-active agents, are in themselves effective tumor promoters when applied often enough to the mouse skin (*12*).

It is the object of the present study to demonstrate by light microscopy and also histoquantitatively (1) that under controlled conditions, laboratory-synthesized dipole-type compounds produce related changes in the epidermis of the back of the mouse; (2) that these changes completely differ from those produced by locally applied carcinogenic hydrocarbons and are positively correlated, in degree, to the respective tumor-promoting property (*11*); and (3) that the changes are, in general, closely related to those caused by a "nonspecific tumor promoter," oleic acid.

MATERIALS AND METHODS

All general procedures were similar to those used in our previous studies.

The experimental animals were white male mice of the stock strain used in this laboratory since 1945 (*1–9*). Our mouse strain is fairly resistant to tumors, and so far no spontaneous tumors have been observed. The animals were 2 to 3 months old at the start of the experiments. They received food and water *ad libitum*. Animals treated with carcinogens were kept in separate laboratory rooms. No mice were lost during the experiments.

Carcinogens.—9,10-Dimethyl-1,2-benzanthracene (DMBA)[4] and 20-methylcholanthrene (MCA)[5] (obtained from L. Light & Co., Colnbrook, England) were dissolved in reagent grade acetone; dosages: experiment A, single dose of 60 γ of carcinogen ("application"); experiment B, 60 and 0.06 γ, respectively, applied continuously once daily, Sundays excluded. Application was made carefully with a glass pipette.

[4] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.
[5] *Chemical Abstracts'* nomenclature: 3-methylcholanthrene.

Secondary treatment.—The technical (t.) surfactants (Atlas Powder Co., Wilmington, Del.) were: Span 60 (sorbitan monostearate)—no tumor-promoting property (*5, 13*): Tween 60 (polyoxyethylene sorbitan monostearate)—an effective tumor promoter (*2, 5, 6, 13–15*); Span 20 (sorbitan monolaurate)—a rather weak tumor promoter (*6*); and Tween 20 (polyoxyethylene sorbitan monolaurate)—a poor tumor promoter (*5, 13*). Besides their various homologues, these technical detergents contain other fatty acids in addition to those stated by the manufacturer. The pure polyol fatty acid esters which were synthesized (s.) by us and which imitated the technical products were: Span 60 (sorbitan monostearate); Tween 60, derived from s. Span 60 by adding about 20 ethylene oxide units; Span 20 (sorbitan monolaurate); and Tween 20, derived from s. Span 20 [*see* (*8*)]. Of these compounds, s. Tween 60 [hydrophile-lipophile balance (HLB) value 14.9] and s. Tween 20 (HLB 16.7) are water soluble; s. Span 20 (HLB 8.6) is dispersible in water, and s. Span 60 (HLB 4.7) insoluble in water. Our intention had been to work with equimolar concentrations calculated from a 25 percent aqueous solution of s. Tween 60. However, owing to the nature of the compounds used, the molar concentrations are approximate only : 0.18 M. Treatment was given by painting the skin with a #16 hair brush, 6 and 12 times a week, continuously, Sundays excluded.

In experiment C the secondary treatment was given without the initial carcinogen application, and in experiment D secondary treatment was started 30 days after application of 60 γ MCA. In experiment E (without initial application of carcinogen) undiluted oleic acid (May & Baker Ltd., Dagenham, England) was applied 6 times a week, Sundays excluded. The treated area on the back of the mouse was the same as that in our earlier experiments.

Histologic and histoquantitative techniques.—The skin biopsy technique has been described (*2–5*). The samples were taken between 8 and 9 a.m. on the 2d, 6th, 10th, 16th, and 30th days after the first treatment. In experiment D (local application with MCA) the same procedure was followed but the first biopsy was taken on the 30th plus 2 days after the application. On each biopsy day, samples were taken from 5 different mice in each series. Specimens for electron microscopic examination were prepared at the same time (report to be published later). The control series comprised 100 untreated animals.

The specimens were fixed in Susa mixture, embedded in paraffin, and stained with hemalum and eosin. The cell count, which has been previously described, was made from the interfollicular area proper; the vicinity of the follicular openings was carefully avoided. Cells in the stratum malpighii were counted from 15 fields per specimen. Every field was 90 μ long so that the whole length, accordingly, was 1350 μ — microscopic magnification $\times$ 1,440 (oil immersion). The following cell types were differentiated: (1) basal cells, (2) differentiating cells, (3) degenerating cells, and (4) mitotic cells.

RESULTS

Normal Interfollicular Epidermis of the Back of the Mouse

The interfollicular epidermis (IFE) of the back of the male mouse is composed of 1 or, at the most, 2 layers of cells surmounted by a relatively abruptly appearing narrow band of keratin (fig. 1). Most of the cells are in contact with and adhere to the dermo-epidermal junction, which appears as a slightly undulating line. The cytoplasm is basophilic, the nuclei are round or oval, and mitotic figures appear only in scattered cells. The intercellular spaces cannot be visualized under the light microscope. In the flattened superficial cells, small amounts of keratohyaline granules stained with hemalum and eosin. The microscopic appearance of the IFE was uniform in all the 100 control animals [for the structure of dermis and cutaneous appendages see (*2, 4, 5*)].

Histoquantitative analyses revealed that in the counted area the mean total number of cells was 243 ± 2.2, excluding stratum granulosum. They consisted of 172 ± 1.9 (71.1 ± 0.46%) basal cells, 61.7 ± 1.6 (25.4 ± 0.46%) differentiating cells, 8.44 ± 0.65 (3.48 ± 0.27%) degenerating cells, and 0.15 (about 0.06 ± 0.02%) mitotic cells. The mitoses were located exclusively in the basal-cell layer.

Experiment A: Single Application of 60 γ of MCA and of 60 γ of DMBA

Since the time of appearance of changes in the IFE depended on the product used for treatment, the observations are divided into stages depending on the elapsed time after the first treatment.

On the 2d day (fig. 2) the IFE was 2 to 3 times thicker than normal, but the number of cells had not increased though they were greatly increased in size; the nuclei were enlarged, and the cytoplasm exhibited swelling, vacuolation, and pallor. The nuclear size varied considerably and many nuclei were karyolytic, while the chromatin was irregularly distributed, frequently along the nuclear membrane. Distinct intercellular bridges had developed with wide intercellular spaces. The amount of keratohyaline granules had increased here and there. On the 6th day (fig. 4) the cell proliferation in the IFE had increased and the cells were somewhat more regular in form. The basal-cell layer could be distinguished from the prickle-cell layer, which had larger cells. A few nuclei were pyknotic. The nuclear size and the amount of chromatin varied from cell to cell. Mitotic figures, in addition to those appearing in the basal-cell layer, were scattered in other layers as well. Stratum granulosum was uneven. In all animals the IFE was transformed into a hyperplastic and hyperkeratotic state, though the hyperkeratosis was irregular. From this day the IFE was markedly thicker and the total number of cells was greater in the DMBA than in the MCA series. By the 10th day the epidermal hyperplasia had distinctly increased in the DMBA series. In the MCA series the degree of hyperplasia showed no clear difference when compared with the status on the 6th day. In

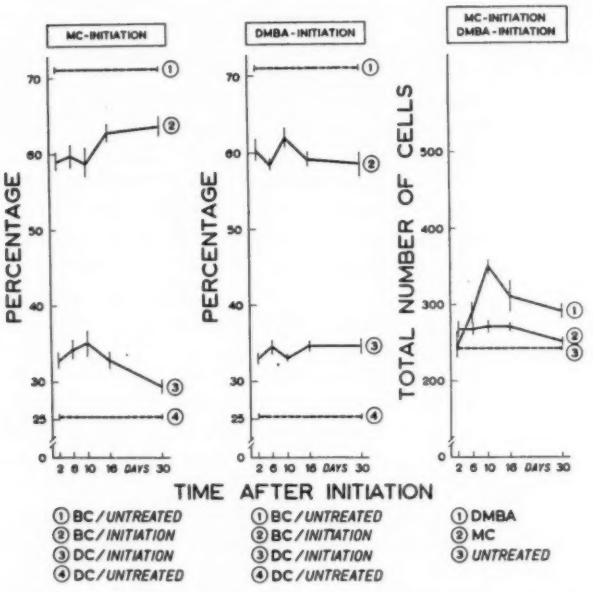
general, the changes in the DMBA and MCA series were reduced by the 10th day: The cells were smaller, and the nuclei uniform and more darkly stained, but the stratum granulosum and stratum corneum were thick. On the 16th day the changes in both series had decreased: In the MCA series the cell size had nearly reverted to normal; stratum granulosum was only present here and there. The DMBA series was clearly different: All layers in the IFE could still be differentiated. On the 30th day (figs. 3 and 5) a further decrease in the changes was seen. However, animals that received this application of DMBA and MCA clearly differed from the controls: Stratum corneum was essentially thicker than that in controls but stratum granulosum was hardly detectable. The cells in stratum malpighii were small, with poorly defined boundaries. Although the chromatin took a strong stain, the amount was variable in different nuclei.

Typical of the DMBA and MCA series were destructive changes in the follicles, which were present on the 2d day. The hair sheaths were hyperplastic, and keratotic plugs were seen in the dilated follicular openings. The sebaceous glands had almost completely disappeared by the 6th and 10th days, and numerous follicles were epilated. Alterations in the pilosebaceous apparatus were clearly more severe in the DMBA series. This was also true at the later experimental stage, though the changes in the pilosebaceous apparatus recovered with time. On the 30th day the openings of only a few follicles were dilated and keratinized, and the sebaceous glands began to regain a more normal appearance. The dermis showed an inflammatory reaction and this also decreased with time.

Histoquantitative analyses (text-fig. 1) revealed, on the 2d day, a nearly normal total cell number: MCA, 268 $\pm$ 13, and DMBA, 248 $\pm$ 20. On the 6th day the total number of cells in the DMBA series had increased; it was highest (350 $\pm$ 11) on the 10th day and decreased thereafter. In the MCA series, again, the total cell number had increased only slightly—on the 30th day it was nearly normal (252 $\pm$ 6.9). The number of differentiating cells was relatively higher than that of the basal cells, and the amount of degenerating cells varied considerably.

Experiment B: Continuous Treatment (6 Times a Week) With 60 and 0.06 γ of MCA and DMBA

Carcinogen per dose, 60 γ.—On the 2d day both series (MCA and DMBA) showed a large increase in the size of cells and nuclei, but with variations in size, especially in the DMBA series. Intercellular spaces had formed which were irregular; the nuclei were vesicular and pale. Distribution of chromatin was irregular, frequently along the nuclear border. Abundant karyolysis was also present. By the 6th day (fig. 6) a relatively high-degree IFE hyperplasia had developed. Its various cellular layers were distinct though irregular in form. Nuclear size varied greatly, but chromatin distribution was more regular. No karyolysis was seen. In some places most cells were a basal type; there were moderate amounts of mitotic cells. On the 10th and 16th days marked disorganization was



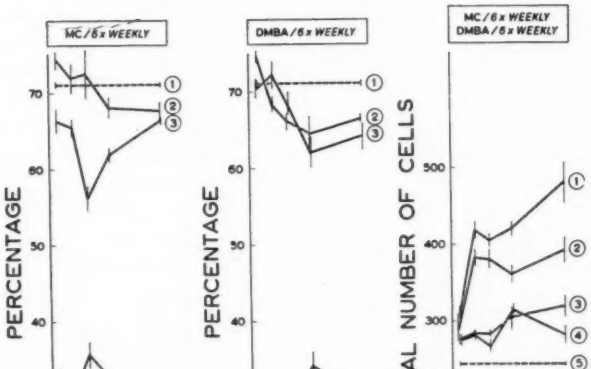
TEXT-FIGURE 1.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: single application ("initiation") of 60 γ MCA and of 60 γ DMBA. (*Vertical lines:* mean $\pm$ = S. D. of the mean.)

conspicuous particularly in the DMBA series. Cellular irregularities (fig. 8) were considerable in both series. There were, in some cells, completely atypical, very large, and/or irregularly shaped nuclei. Pathologic mitoses were also present. Intercellular spaces were wide and irregular. In the DMBA series, especially, the IFE was again difficult to distinguish from the enlarged and greatly hyperplastic follicles. On the 30th day, with DMBA (fig. 7), the amount of irregular hyperplasia had increased further. The altered IFE formed, with the cells of follicular origin, large, irregular extensions and vegetation downward into the dermis. The cells in these extensions showed a disorderly arrangment; there also was moderate atypia and, in some places, dyskeratosis. In the MCA series, also (fig. 9), the cell damage and general irregularity of the IFE were accentuated, but not to the same extent as in the DMBA series. The thickness of the stratum granulosum varied considerably. Here and there the epidermal surface was parakeratotic. The stratum corneum was usually abundant.

As the treatment period progressed, the follicles exhibited progressively increasing destructive alterations that were connected with follicular hyperplasia. From the 6th day nearly all the sebaceous glands had disappeared or they appeared as deformed remnants. The follicular openings were dilated, increasingly filled with keratin plugs, and, in most instances,

totally epilated. On the 30th day the deformed follicles in many places in the corium formed irregular cysts filled with horny material. From the beginning there were marked inflammatory alterations throughout the corium. Later the upper corium, especially, appeared rather degenerated—it was edematous, somewhat homogenized, and had few cells. The changes in the lower corium seemed more exclusively inflammatory.

Histoquantitative analyses (text-fig. 2) revealed that on the 2d day the total number of cells had slightly increased from normal: MCA, 291 ± 18, and DMBA, 307 ± 9.6. But from the 6th day the cell count disclosed a



TEXT-FIGURE 2.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: 60 γ (as 0.3% acetone solution), and 0.06 γ (as 0.0003% acetone solution) of MCA and DMBA, 6 times a week.

high-degree hyperplasia: MCA, 382 ± 12, and DMBA, 419 ± 14. Both the absolute number of basal cells and that of differentiating cells had greatly increased. Their numerical ratio therefore differed very little from that in the normal IFE. The percentage of degenerating cells was rather high; *e.g.*, on the 30th day: MCA, 5.9 ± 0.47, DMBA, 5.6 ± 0.93. The percentage of mitotic cells was highest in the MCA series on the 10th day (0.6 ± 0.1%) and in the DMBA series on the 6th day (0.5 ± 0.1%). Mitotic figures, in addition to those appearing in the basal-cell layer, were scattered in other layers also. On the 30th day the total number of cells

in the MCA series was 392 ± 18 and in the DMBA series, 480 ± 28. The degree of hyperplasia had progressively increased.

Carcinogen per dose, 0.06 γ.—On the 2d day the IFE showed, in some places, a definite though low-degree increase in cell size. On the 6th day (figs. 10 and 12) there were areas in the IFE in which the cells were much above normal in size; the total number of cells, however, was nearly unchanged. The intercellular spaces in these areas were distinct. The basal- and prickle-cell layers were distinguishable and developed like the stratum spinosum and the thin stratum granulosum; the stratum corneum was thicker than normal. The nuclei stained intensely. Differences were observed between the two series in that the increase in cell size and the general increase in the IFE thickness were greater in degree in the DMBA series. Typical of both series were regional variations in the intensity of the alterations. On the 10th day the situation was similar to that on the 6th day. On the 16th day the IFE was hyperplastic here and there. On the 30th day (figs. 11 and 13) the variations in size and staining of nuclei had increased further, though cells generally were relatively small. In many places there was hyperkeratosis.

The follicles exhibited comparatively few changes, which appeared chiefly on the 16th day. Some follicular openings had keratin plugs. In the upper corium there were only slight inflammatory reactions.

Histoquantitative analyses (text-fig. 2) revealed that on the 2d day the total number of cells had only slightly increased from normal: MCA, 274 ± 7.3, and DMBA, 277 ± 7.3. On the 16th day the hyperplasia was marked: MCA, 315 ± 11, and DMBA, 305 ± 17. The percentage of mitoses was manifold compared with the normal: MCA, 0.4 ± 0.2, and DMBA, 0.4 ± 0.2, on the 30th day. Mitotic figures, in addition to those in the basal-cell layer, were sometimes observed in more superficial layers. With these low doses of carcinogen, the difference in potency between MCA and DMBA is not so clear.

Experiment C: Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Tween 60, s. Span 60, and s. Tween 20, Without Preceding Carcinogen Initiation

Changes in IFE produced by other technical products of this group have been reported earlier (*2, 4, 5*). For this reason the experiment was repeated only with the most potent compound, t. Tween 60. The effect of Span 20, the parent compound of Tween 20, is analyzed in paper II (*16*).

T. Tween 60 and s. Tween 60.—These two compounds had a similar effect on the IFE. On the 2d day the IFE had very greatly thickened; the number of cells increased and they were arranged in 3 or 4 layers. Cells and their nuclei were large and stained lightly—nuclei were vesicular, and chromatin was regularly arranged. Many cells showed perinuclear vacuolation, which was greatest in the series treated 12 times a week (2 times a day) with s. Tween 60. The intercellular spaces were wide, and they as well as the well-differentiated intercellular bridges were uniform. Some of the basal cells were of a spinous type. Parakeratosis was present

especially in the series treated 12 times a week. On the 6th day the number of cells showed further increase. In general the alterations were similar, but the different cell layers were more distinctly discerned. The nuclei of the basal cells were more intensely stained than those of the prickle-cell type and they had less cytoplasm. In some of the specimens the stratum granulosum contained keratohyaline granules, and in some there was parakeratosis. Intracellular vacuolation was seen chiefly in the series treated 12 times a week. On the 10th, 16th, and 30th days very little of this remained, but the intercellular spaces continued to be wide. The thickness of the stratum granulosum and stratum corneum was related to the amount of hyperplasia, though many specimens still showed parakeratosis (figs. 14 and 15). The size of the cells and nuclei varied comparatively little through the period of experiment. No pathologic mitosis was seen. There were occasional pyknotic nuclei, but other damage to cells or nuclei was not seen.

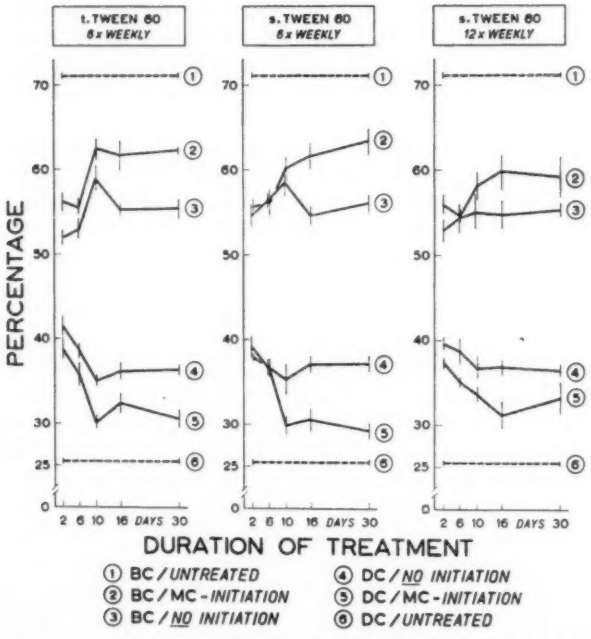
In many instances the hyperplasia of the IFE extended to the upper portion of the follicles; the follicular openings were then slightly dilated and keratinized. The general shape of the follicles nevertheless was regular, and the hair was intact. The follicles were slightly larger than normal, independent of the phase of hair growth, and more than half the follicles were in the growth phase (*cf. 2, 4, 5*). From the 2d day the dermis was edematous, and throughout the experimental period there were diffusely distributed inflammatory cells.

Histoquantitative analyses (text-figs. 3 and 5) revealed that on the 2d day the total number of cells had greatly increased in all series but most in the s. Tween 60 (6 times a week): 399 $\pm$ 8.0. On the 6th day the thickening of the IFE was still more marked. Thereafter the total number of cells remained at approximately the same level in all series other than that treated 12 times a week in which it had increased further; the total number of cells was highest on the 30th day: 484 $\pm$ 26. The hyperplastic IFE was composed chiefly of differentiating cells, whereas the basal cells had hardly increased. The percentage of degenerating cells was fairly constant (from 5 to 7%). The percentage of mitotic cells increased throughout the experimental period, but was highest on the 2d day: t. Tween 60, 1.7 $\pm$ 0.2, and s. Tween 60, 1.3 $\pm$ 0.3 percent. Mitotic figures appeared only in the basal-cell layer, and were never observed in other layers of the hyperplastic IFE.

S. Span 60 and s. Tween 20.—On the 2d day, in the s. Span 60 series, a slight thickening was observed in the IFE. Cells and nuclei, however, were fairly normal in size or slightly larger. In the two series treated with s. Tween 20 (treatment 6 and 12 times a week) the IFE had thickened, and the cells and their nuclei had increased in size; there were narrow intercellular spaces and occasional intercellular bridges were visible. A narrow stratum granulosum was also discerned. On the 6th day the IFE of a few animals treated with s. Tween 20 was clearly hyperplastic. In the s. Span 60 series there was no change from the situation of the 2d day. On the 10th day the IFE in the s. Tween 20 (12 times a week) series

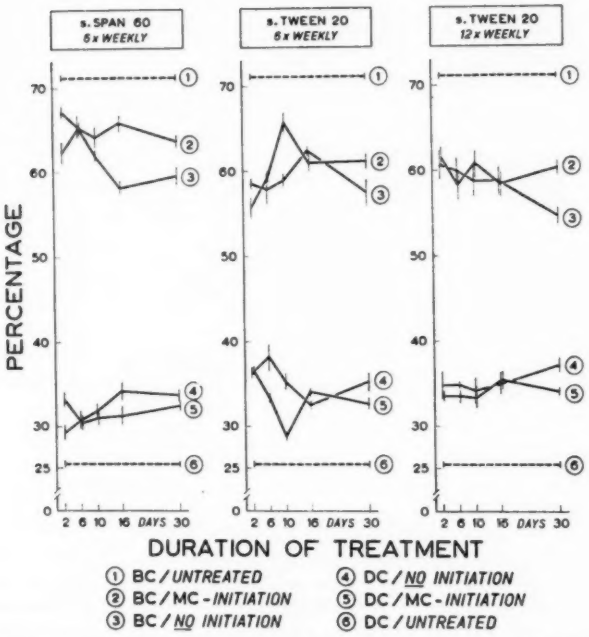
was still slightly hyperplastic, but in the series treated 6 times a week, the epidermal reaction had become weaker and was approximately similar to that in the s. Span 60 series. In the latter, the cells were relatively small, but in the s. Tween 20 series they were slightly larger. On the 16th and 30th days (figs. 18 and 20) the appearance of the IFE was practically unchanged.

Histoquantitative analyses (text-figs. 4 and 5) revealed that the total number of cells in the IFE had not increased to any noteworthy degree in the s. Span 60 series during the entire treatment period. In the two s.



TEXT-FIGURE 3.—Percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: t. and s. Tween 60 with and without initial MCA application.

Tween 20 series, the total number of cells had definitely increased on the 2d and 6th days. Thereafter the number of cells in the s. Tween 20 (12 times a week) series increased throughout the experimental period; and in the s. Tween 20 (6 times a week) series the total cell number declined to nearly normal. Despite the small change in the total number of cells, there was a larger number of differentiating cells than in the normal IFE. The percentage of degenerating cells was below normal. Mitotic cells had increased in number, which was greatest in the s. Tween 20 series treated 12 times a week. The number of mitotic cells was not nearly as high in any of these series as in the t. and s. Tween 60.



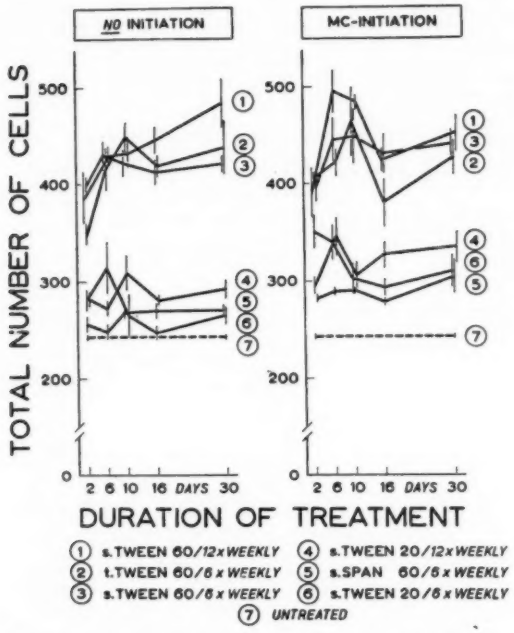
TEXT-FIGURE 4.—Percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: s. Span 60 and s. Tween 20 with and without initial MCA application.

Experiment D: Continuous Local Treatment (6 and/or 12 Times a Week) With t. and s. Tween 60, s. Span 60, and s. Tween 20, After Initial Local Application of 60 γ of MCA

T. and s. Tween 60.—The effect of these two compounds on the IFE, pilosebaceous apparatus, and dermis was similar.

The histologic appearance differed completely from that found to be characteristic of the effect of these agents when used without an initial carcinogen application (expt. C).

When a secondary treatment with t. and s. Tween 60 was begun 30 days after an application of MCA, the IFE, on the 2d day, was hyperplastic to a high degree and consisted of 3 to 4 layers of cells. The cells and their nuclei had increased in size, and there was cellular and nuclear atypia. Cytoplasm and nuclei stained more intensely and were more basophilic than in the series without an initial application. Intracellular vacuolation was not seen; the intercellular spaces were wide and the intercellular bridges distinct; parakeratosis appeared occasionally; and the diameter of stratum granulosum varied. On the 6th day the thickness of the cell layers had increased further. The comparatively small, intensely stained basal cells now formed several layers here and there, with only a small number of larger-size prickle cells situated above. In some places



TEXT-FIGURE 5.—Development and degree of hyperplasia (alterations in the total cell count) in IFE. Treatment: t. Tween 60, s. Span 60, s. Tween 60, and s. Tween 20 with and without initial MCA initiation.

there was hypergranulosis and hyperkeratosis, in others there were areas in which the changes resembled those produced by these compounds alone; parakeratosis was uneven. In some cells there was also intracellular vacuolation, though very slight in amount. Through the 10th, 16th, and 30th days the degree of hyperplasia remained nearly unchanged. Edematous changes, however, had decreased. In extensive areas of the IFE, basal cells predominated (figs. 16 and 17).

In these series the follicles also exhibited various alterations. The IFE hyperplasia extended fairly deep; the openings of numerous follicles had keratin plugs; and part of the follicles were epilated. The dermis had thickened and showed mild inflammatory changes.

Histoquantitative analyses (text-figs. 3 and 5) revealed that in all series there was a high-degree increase in the total cell number. This was highest on the 6th and 10th days after secondary treatment had begun. However, the total cell number was at the same level as in the series without an initial application of MCA. Cell counting showed, in all the MCA series, a great increase in the number and percentage of basal-type cells compared with the series treated with t. and s. Tween 60 alone. The percentage of mitotic cells in the noninitiated series (expt. C) was somewhat higher: mean, 0.93 percent, while in the series treated with the same agents after initiation, the mean was 0.78 percent. In contrast to those

series mentioned, which were treated without an initial application of carcinogen, mitotic figures *now* appeared also in the more superficial layers of the hyperplastic IFE. The percentage of degenerating cells in the MCA series varied from 4 to 10 percent.

S. Span 60 and s. Tween 20.—In contrast to the preceding, the changes differed comparatively little from those seen in the series without MCA (expt. C). In the s. Span 60 series, only scanty changes were found in IFE compared with those present before the secondary treatment. The thickness of IFE had only slightly increased. The cellular size was nearly normal (*cf.* figs. 18 and 19). In the s. Tween 20 series there was a low-degree IFE hyperplasia by the 2d day, and the cells had clearly increased in size. Intercellular spaces, although narrow, were visible. They persisted practically unchanged to the 6th, 10th, and 16th days and, partially, to the 30th day. The stratum corneum was thicker than normal throughout this time. In contrast to the noninitiated series, the changes by the 30th day varied from area to area, *i.e.*, among others, the size and staining of nuclei and the thickness of stratum corneum (*cf.* figs. 20 and 21).

The follicular openings were in some places dilated and contained some keratin plugs. The dermis was somewhat thickened and here and there contained inflammatory cells.

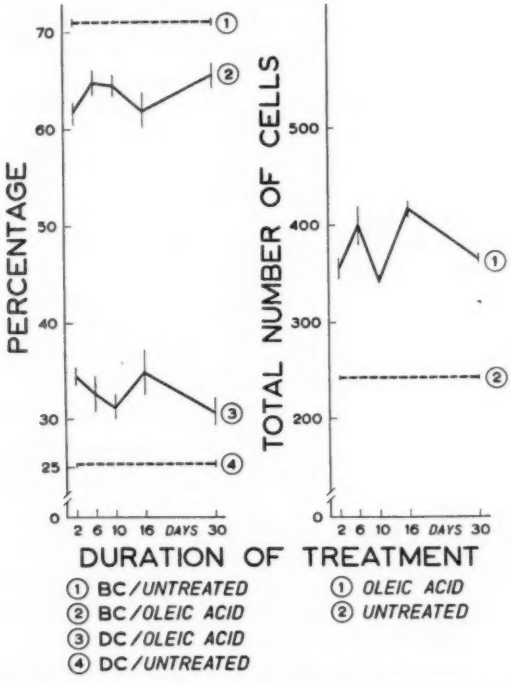
Histoquantitative analyses (text-figs. 4 and 5) revealed that the total cell number in the stratum malpighii had increased in all series and most in those given the secondary treatment 12 times a week with s. Tween 20. The total number of cells in the IFE was slightly higher than in the corresponding noninitiated series. The percentage of basal cells had greatly increased in the initiated series, though differences were slight. There was no difference in the percentage of mitotic or degenerating cells.

Experiment E: Continuous Local Treatment (6 Times a Week) With Undiluted Oleic Acid Without Initial Carcinogen Application

Certain general features of the skin changes produced by this fatty acid after carcinogen application have been described (*17*). The nature of the hyperplasia produced by undiluted oleic acid alone will be analyzed here. On the 2d day the IFE was hyperplastic, the cells were greatly enlarged, and in some places the cytoplasm was swollen and pale. The nuclei had increased in size, and chromatin was normally distributed within the nuclei. Mitotic figures, moderately present, were normal in structure. Intercellular spaces had formed, and intercellular bridges were well differentiated. In some places there was parakeratosis, in others the stratum granulosum and stratum corneum were regularly formed. On the 6th day the degree of hyperplasia had somewhat increased. Cell layers were regularly formed, basal cells were smaller than prickle cells, and the nuclei stained uniformly. On the 10th (fig. 22), 16th, and 30th days (fig. 23) the degree of hyperplasia was approximately as before, only some variation in thickness of the IFE was seen from animal to animal. In some mice the stratum granulosum was remarkably thick.

Some follicular openings were dilated and contained keratin plugs; epilation did not develop; and sebaceous glands were normal. The dermis exhibited a mild inflammatory reaction during the entire experimental period.

Histoquantitative analyses (text-fig. 6) revealed, by the 2d day, a relatively marked hyperplasia. The highest total cell count was seen on the 16th day: 417 ± 9.2. The percentage of differentiating cells had increased greatly from normal, and the percentage of mitotic cells was moderately high: 0.8 ± 0.1 percent, on the 2d day. The mitoses were located exclusively in the basal-cell layer of the hyperplastic IFE. There were few degenerating cells.



TEXT-FIGURE 6.—Development and degree of hyperplasia (alterations in the total cell count), and percentage distribution of basal cells (BC) and differentiating cells (DC) in IFE. Treatment: undiluted oleic acid, without initial carcinogen application 6 times a week.

DISCUSSION

The primary changes produced by agents that influence skin-tumor formation may be of a different type than the changes that develop later. The alterations may be transitory, permanent, or progressive. For evaluation of the nature and significance of the cutaneous changes, it is

definitely necessary to observe their development *over a long period.* The *nature* of epidermal hyperplasia must be studied to derive adequate results.

The IFE of the back of the mouse is an excellent target tissue. It is composed of one—at the most two—layers of cells, and most cells are undifferentiated (*4, 5*). The mice of the strain used in this laboratory are well suited for the purpose, since their skin has no features that may be correlated to tumor formation. The changes produced by each test compound were, in general, the same in all animals.

It has been reported previously (*9*) that products of Span 60 (sorbitan monostearate), Tween 60 (polyoxyethylene sorbitan monostearate), Span 20 (sorbitan monolaurate), and Tween 20 (polyoxyethylene sorbitan monolaurate), which we synthesized under controlled conditions, resembled the corresponding technical products in their tumor-promoting property or lack of it.

It has now been demonstrated that the synthesized products also produce fully related cutaneous changes. The "Tween 60 effect" (Tween 60 was obtained by adding about 20 ethylene oxide units to inactive s. Span 60) is not a result of by-products of the synthesis. Span 20, which contains no ethylene oxide chains, has, in itself, a moderate tumor-promoting property (*6*). But when a product of the type of Tween 20 is prepared from it, the tumor-promoting property, as well as the hyperplasia-causing effect of the resulting product, decline.

The effect of a locally applied compound of the Tween 60 type completely differs from the skin changes produced by carcinogens applied in the same manner, *i.e.*, MCA and DMBA. These differences are demonstrable also by a rough, light-microscopic technique, and the following are stressed:

1. A single application of 60 γ of carcinogen produced, immediately, skin changes of a toxic nature, including severe cell and nuclear damage. Reparative cell proliferation began only after these changes had become quiescent. Similar treatment with a Tween 60-type product did not cause retrogressive changes; intense cell proliferation began immediately in the IFE. The same was true of a high-degree hyperplasia (text-figs. 1 and 5).

2. Continuous treatment with a carcinogen (60 and 0.06 γ, respectively, 6 times a week) produced changes in the IFE that were greater, more effective, and/or longer than the duration of the action of the carcinogen. A severe degree of cell atypia developed and abnormal nuclei and progressive disturbances in the organization and differentiation of the cells. The percentage of compactly built basal-type cells increased progressively, with a relative decrease in differentiating cells. The percentage of mitotic figures increased considerably. Pathologic mitoses were abundant. Mitotic figures were also observed in the more superficial layers of the hyperplastic IFE. Hyperplasia was absent on the 2d day, but it developed thereafter and increased progressively throughout the treatment period (text-fig. 2). When the dose of carcinogen was low (0.06 γ) this progressive increase in proliferation was not manifested to any noteworthy

degree, though there were definite changes in cells and nuclei. Similar treatment with a Tween 60-type product (an effective tumor promoter) did not cause cell or nuclear atypia in the IFE, but the size of cells was clearly and uniformly increased. A strong intercellular edema developed, especially in the basal parts of the IFE, and well-differentiated intercellular bridges appeared. Further typical changes were a strong intracellular edema and perinuclear vacuoles, which had previously been found typical of the corresponding technical products (*2, 4, 5*). The percentage of differentiating cells increased evenly and greatly, with a relative decrease in basal cells. The number of mitoses was high, but no pathologic mitoses were seen. All mitoses appeared in the basal-cell layer. A high-degree hyperplasia developed immediately. Cell layers were uniformly and regularly arranged. The amount of hyperplasia increased up to the 16th day; treatment thereafter merely maintained it unchanged (text-figs. 3 and 5). The alterations did not change essentially when the applications were doubled (12 times a week). The slight but immediately appearing changes due to products of the Span 60 type (no tumor-promoting effect) and Tween 20 (weak tumor promoter) remained unchanged or were, at the end of the observation period, even less pronounced (Tween 20) than the first reaction had been (text-figs. 4 and 5).

3. A similar treatment with carcinogen, as mentioned in paragraph 2, and even the initial application alone, produced follicular destruction, epilation, partial or total disappearance of the sebaceous glands, and, in the root sheaths, changes similar to those in the IFE. Definite degenerative changes developed in the dermal components. Similar treatment with a Tween 60-type product produced mild hyperplasia in the pilosebaceous glands, which remained completely unchanged when the frequency of treatment was doubled. The dermis increased in thickness and exhibited edema and a uniform mild inflammation.

4. The nature and degree of skin changes that developed after a single initial application of carcinogen as well as during continuous treatment with carcinogen varied essentially even in different parts of the treated area. Changes produced by a Tween 60-type product were similar throughout the whole area.

5. Under experimental conditions, MCA was better suited for these studies than DMBA—MCA initiation produced a comparatively low-degree hyperplasia. The thickness of the IFE reverted to nearly normal in 30 days (text-fig. 1). Experiment D was specially constructed on the basis of this principle (*see* 6).

6. The cutaneous changes produced by continuous treatment (6 times a week) with a Tween 60-type product, which began after an interval of 30 days from the initial application of MCA, differed essentially from those produced by Tween 60 alone (*cf.* figs. 14 and 16). Cell and nuclear atypia developed. The cells of the IFE clearly exhibited increased resistance to the capacity of a Tween 60-type tumor promoter to produce intracellular edema (*cf.* 2). There was a marked tendency to keratinization; widened follicular openings had keratin plugs, etc. The percentage

of basal-cell-type elements increased significantly compared to differentiating cells (text-figs. 3 and 5). The effect of a single initial application of MCA was manifested not only in these structural changes but, above all, in a retardation and disturbance of the maturing of the IFE cells, when cell multiplication was forced to start *afterward* by secondary treatment, and was continuously maintained with Tween 60. Now mitotic figures were observed in several layers of the hyperplastic IFE. After MCA application, similar treatment with Span 60- and Tween 20-type products, which when used alone only produced a transitory weak hyperplastic reaction, did not manifest the alterations mentioned (figs. 18 to 21). Now the percentage of basal cells increased slightly (text-fig. 4) and the thickness of the IFE increased insignificantly (text-fig. 5).

7. Continuous treatment (6 times a week) with undiluted oleic acid alone (effective tumor promoter) produced persisting IFE hyperplasia (text-fig. 6) which, in principle, was the same as that caused by a Tween 60 product alone (*cf.* 2). Oleic acid, however, slightly affected the pilosebaceous apparatus. The general manner of reaction (figs. 22 and 23) was nevertheless essentially different from that evoked by the carcinogen.

It is characteristic of the action of dipole-type tumor promoters ["cocarcinogenic lipides" (*18*)] that their tumor-promoting property and capacity to produce a persisting epidermal hyperplasia are the more effective the more frequently the skin of a resistant-strain mouse is treated with the compounds, as such, or as aqueous solutions. We have previously stressed that a general benignity (*2, 4, 5, 9*) and a marked tendency to regression (*5, 6*) are characteristic of tumors promoted by Tween 60, and by the known tumor promoters of the most simple type such as certain fatty acids (*17*). This is true also of wound-healing as promoting noxa (*e.g., 19*) and of croton oil (*15*). In some instances the sex of the mouse may be involved, *e.g.*, with Tween 60 (*5, 12*) and oleic acid (*12, 17*).

While seeking new tumor promoters for the mouse skin, by using the "frequent application technique" introduced in this laboratory (*20*), it has become evident that a complete spectrum of them exists, from highly potent to rather weak ones. Some of them are physiologic, which are formed and/or needed by the organism [certain fatty acids (*12, 17*), and glycerol fatty acid esters (*21*)]. These dipole-type, tumor-manifesting agents may be regarded as "true tumor promoters." Biologically it is immaterial whether the tumors result from the use of comparatively large total doses and/or from the use of frequent applications. The same high incidence of tumors can be obtained even with one tenth as large a total dose per animal, provided treatment is given often enough (*5*). The biological result in any case is the manifestation of tumors. Histologically they also fulfill all the criteria of tumors (*4, 5*). Accordingly there develops precisely the phenomenon for which the concept "tumor promotion" originally was created by Friedewald and Rous, 1944 (*19*).

Promotion of tumors in the skin of the back of the mouse can be

produced by numerous polyol fatty acid esters, by many fatty acids, and other dipole-type tumor promoters. In its purest form, tumor promotion caused by dipole-type agents is a process of a fully benign nature. Histologically and histoquantitatively, tumor promotion (in this case) is manifested as an intense and a continuous multiplication (proliferation) of epidermal cells, when the percentage of mitoses may increase tenfold and even 20-fold from the normal. As a result of this the amount of cells in differentiation increases. The condition should be regarded as comparable to "simple" reparative cellular proliferation. This activity merely provides the conditions for the manifestation of the actual carcinogenetic process started by the initiation or, in sensitive mouse strains, even without it. *The tumor-promotion process as caused by dipole-type agents is not a part of the carcinogenetic process itself* [*cf.* also data and discussion, *e.g.*, in (*2*, *4*, *5*)].

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PLATE 86

All photomicrographs. Hemalum and eosin. × 530

FIGURE 1.—Mouse #3086. Normal IFE of back of adult mouse: Cells in 1 to 2 layers, nuclei oval, cell margins indistinct, no intercellular spaces, keratohyaline granules as a scarcely visible band, and thin keratin lamellae at surface.

FIGURE 2.—Mouse #7135. A single initial application of 60 γ of DMBA; no secondary treatment, 2d day: IFE thickened, cells markedly increased in size, intercellular spaces wide and intercellular bridges distinct, nucleus/protoplasm ratio altered, and several enlarged nuclei.

FIGURE 3.—Mouse #7157. A single initial application of 60 γ of DMBA; no secondary treatment, 30th day: Thickness of IFE increased, cells and nuclei rather small, slight irregularity in nuclei, and stratum corneum thicker than normal.

FIGURE 4.—Mouse #7114. A single initial application of 60 γ of MCA; no secondary treatment, 6th day: Low-degree hyperplastic reaction, intercellular spaces narrow, size of nuclei slightly variable, increased chromatin content and increased keratinization.

FIGURE 5.—Mouse #7132. A single application of 60 γ of MCA; no secondary treatment, 30th day: Same alterations as in figure 4, but less pronounced.

FIGURE 6.—Mouse #9111, 60 γ of DMBA, 6 times a week, 6th day: IFE highly hyperplastic, cells irregularly increased in size, intercellular spaces wide and intercellular bridges visible, size and chromatin content of nuclei variable, and stratum granulosum and stratum corneum thick and irregular.

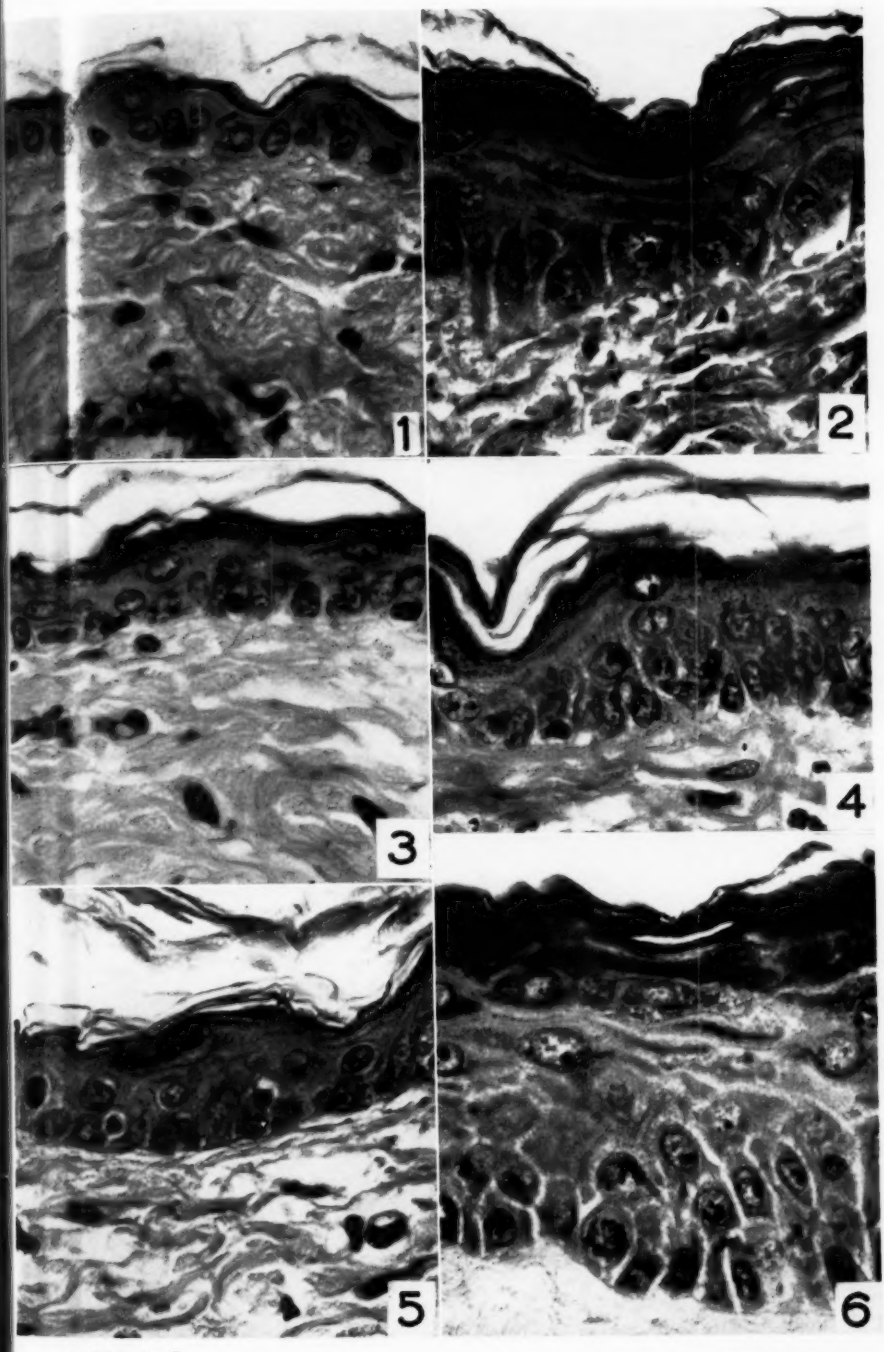

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PLATE 87

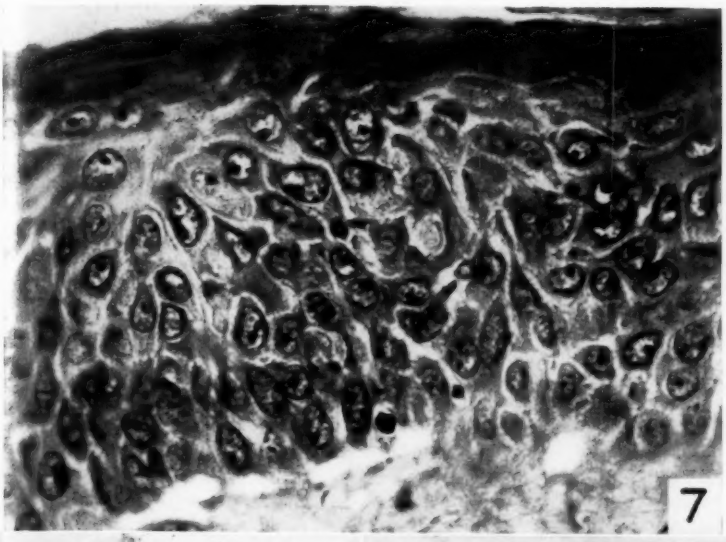
FIGURE 7.—Mouse #9129, 60 γ of DMBA, 6 times a week, 30th day: Extremely high-degree hyperplastic reaction in IFE, cells and nuclei irregular in shape and variable in size, chromatin irregularly localized, nucleoles of different sizes, karyolysis, huge cells, and irregular intercellular bridge formation.

FIGURE 8.—Mouse #9097, 60 γ of MCA, 6 times a week, 16th day: Irregular hyperplasia, marked variations in size of nuclei and cells, and also considerable atypia. (On *left*, a huge nucleus.)

FIGURE 9.—Mouse #9101, 60 γ of MCA, 6 times a week, 30th day: High-degree and irregular IFE hyperplasia; nuclei and cells show great variation.

FIGURE 10.—Mouse #9063, 0.06 γ of DMBA, 6 times a week, 6th day: IFE thicker than normal; size and chromatin content of nuclei variable.

FIGURE 11.—Mouse #9078, 0.06 γ of DMBA, 6 times a week, 30th day: Slightly hyperplastic IFE thicker than normal, variation in nuclei and cells, and hyperkeratosis.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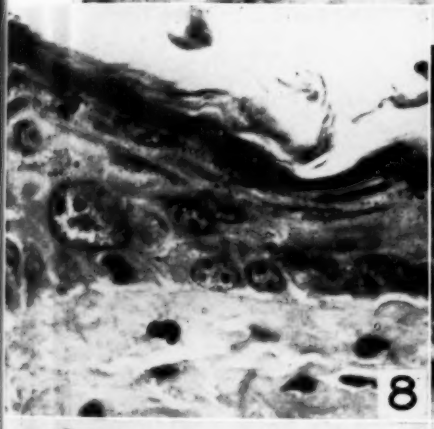
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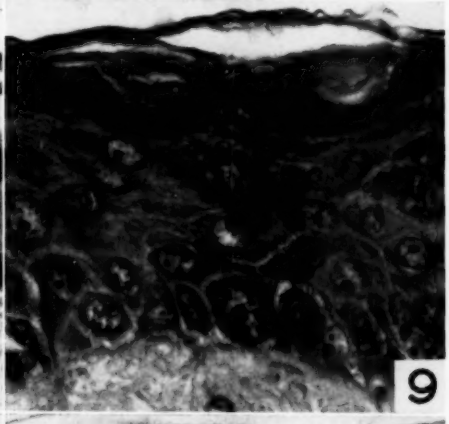
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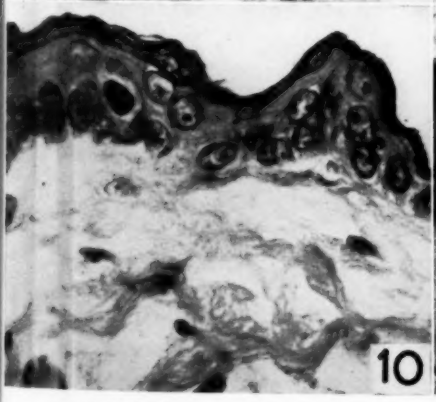
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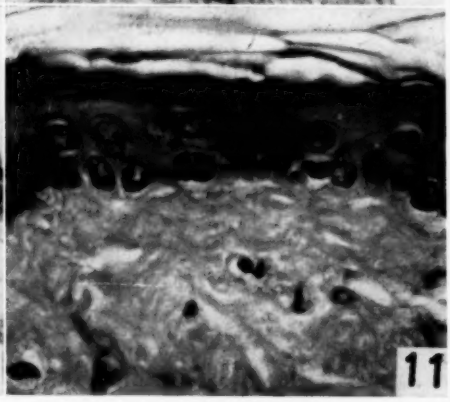
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PLATE 88

FIGURE 12.—Mouse #9036, 0.06 γ of MCA, 6 times a week, 6th day: Changes similar to those in figure 10.

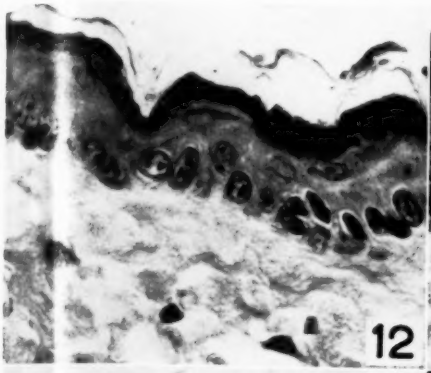
FIGURE 13.—Mouse #9051, 0.06 γ of MCA, 6 times a week, 30th day: Unevenness and irregularity of IFE clearly evident.

FIGURE 14.—Mouse #7207, s. Tween 60, 6 times a week, without preceding initiation, 30th day: IFE even, regular IFE hyperplasia; cells in neat layers, cells and nuclei uniformly increased in size; intercellular spaces wide and intercellular bridges well differentiated, basal cells situated in one row, and differentiating cells abundant (*cf.* figs. 7, 9, 11, and 13).

FIGURE 15.—Mouse #7229, s. Tween 60, 12 times a week, without preceding initiation, 30th day: High-degree, regular IFE hyperplasia. Changes similar to those in figure 14, but intercellular spaces are wide and some pyknotic nuclei are seen; parakeratosis.

FIGURE 16.—Mouse #7357, s. Tween 60, 6 times a week, beginning 30 days after a single initial application of MCA, 30th day: Pronounced, irregular hyperplastic response, nuclei stained dark, and are comparatively small; basal type elements predominate, and number of true prickle cells is small.

FIGURE 17.—Mouse #7366, s. Tween 60, 12 times a week, beginning 30 days after a single initial application of MCA, 30th day: The same as in figure 16, but intercellular spaces markedly widened. Most cells are of basal type with spindle-shaped nuclei (longitudinal axis of cells at right angles to dermo-epidermal junction).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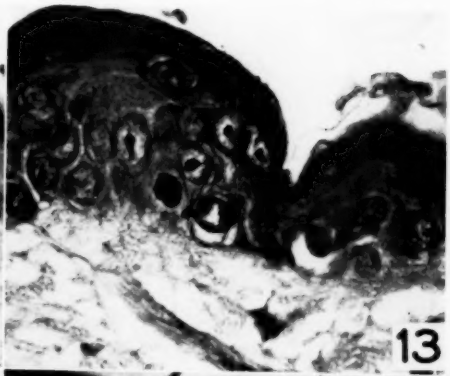
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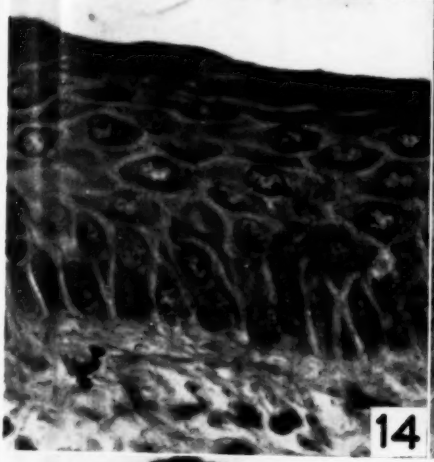
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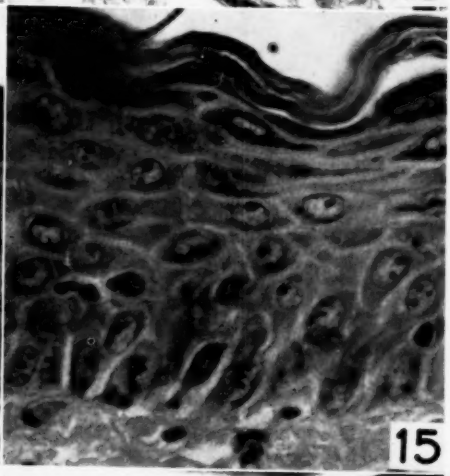
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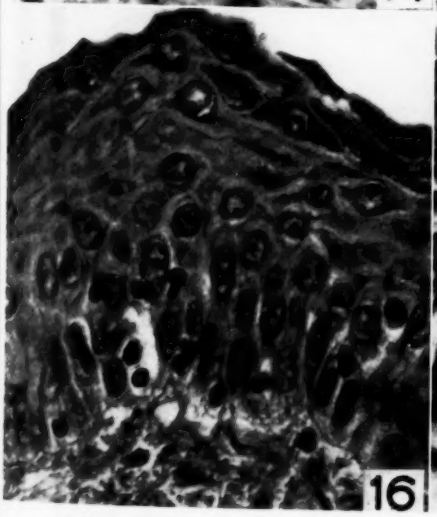
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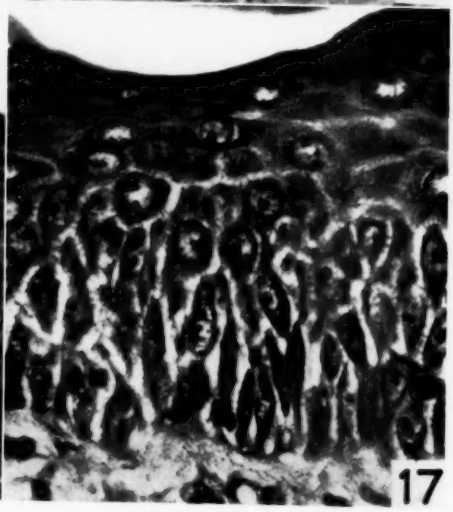
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PLATE 89

FIGURE 18.—Mouse #7258, s. Span 60, 6 times a week, without initial carcinogen application, 30th day: IFE sparsely thickened; very small increase in number of cells (*cf.* fig. 14).

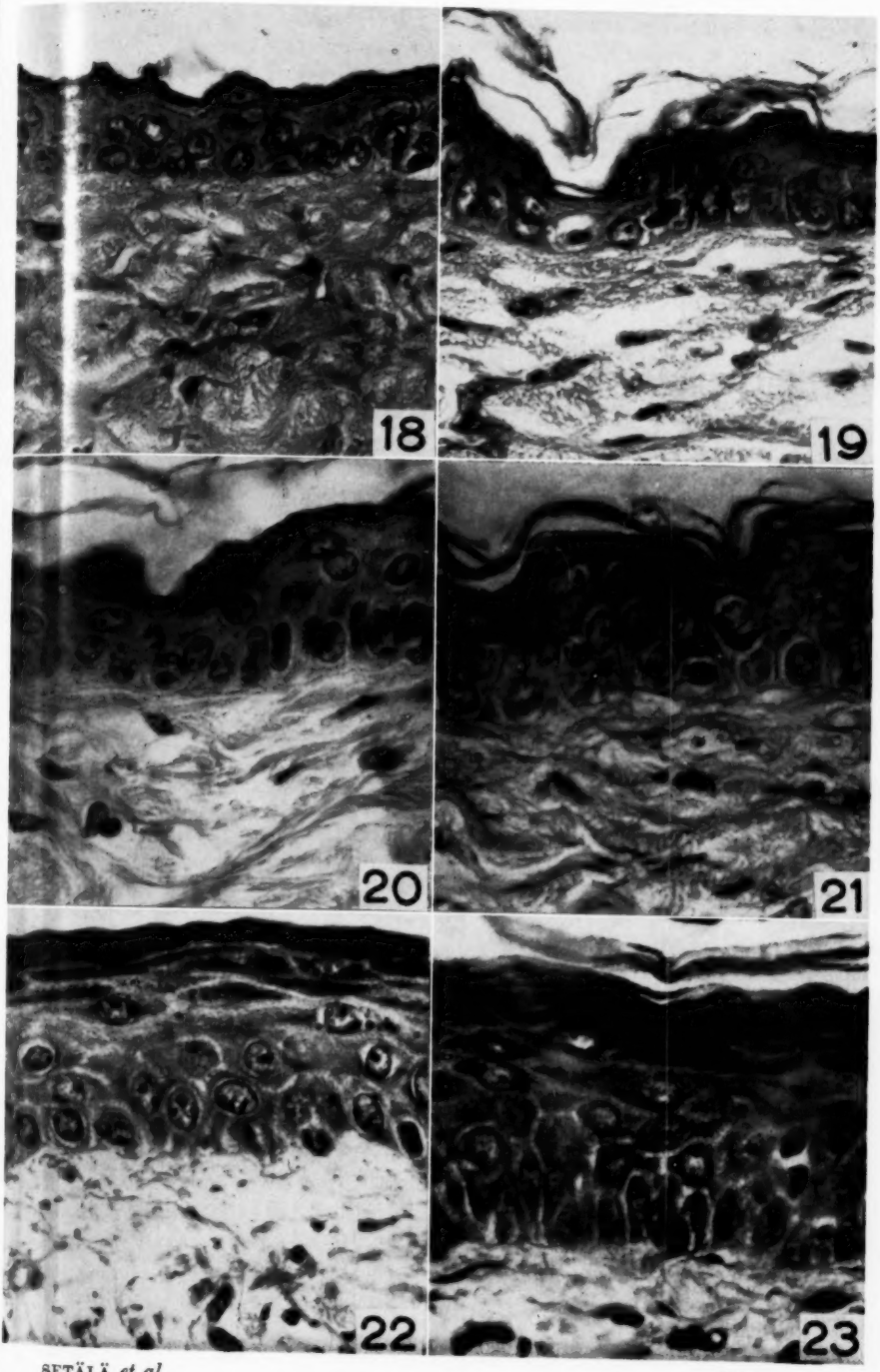
FIGURE 19.—Mouse #7409, s. Span 60, 6 times a week, beginning 30 days after a single initial MCA application, 30th day: Approximately same as figure 18, but possibly slight variation in size of nuclei; stratum corneum is thick.

FIGURE 20.—Mouse #7280, s. Tween 20, 6 times a week, without initial carcinogen application, 30th day: Low-degree IFE hyperplasia. Cells slightly but evenly increased in size.

FIGURE 21.—Mouse #7430, s. Tween 20, 6 times a week, beginning 30 days after a single initial MCA application, 30th day: Some small variations in nuclei; stratum corneum thickened (*cf.* fig. 20).

FIGURE 22.—Mouse #9016, oleic acid, 6 times a week, 10th day: Moderate IFE hyperplasia, cells and nuclei uniformly increased in size, cell layers even; intercellular, although narrow, spaces have formed; intercellular bridges well differentiated.

FIGURE 23.—Mouse #9028, oleic acid, 6 times a week, 30th day: Same as in figure 22, but basal cells situated in one layer slightly smaller. Compared with changes produced by s. Tween 60 (fig. 14) cells are slightly smaller, intercellular spaces narrower, and degree of IFE hyperplasia slightly lower.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Mechanism of Experimental Tumorigenesis. II. Effect of Mole Ratio Distribution of Span 60 and Span 20 Derivatives on Hyperplasia in Mouse Epidermis [1, 2, 3]

KAI SETÄLÄ, LAURI MERENMIES, LEO STJERNVALL, *and* YRJÖ AHO,[4] *Department of Pathology, University of Helsinki, and Department of Chemistry, Institute of Technology, Helsinki, Finland*

SUMMARY

The evolution, general development, persistence, and nature of the hyperplastic response in the interfollicular epidermis of the back of white male mice of the tumor-resistant strain used in this laboratory was followed for 30 days. Ethylene oxide (EO) derivatives of technical Span 60 (sorbitan monostearate) and Span 20 (sorbitan monolaurate) were deliberately selected, which contained 5, 10, 15, 20, 30, 40, 60, and 100 EO units. The skin was painted 6 times a week with approximately 0.18 M aqueous solutions and dispersions of the compounds. Except for compounds containing, on the average, 5 and 100 EO units, which were biologically somewhat weaker, all Span 60 EO derivatives produced a high degree of regular and persisting epidermal hyperplasia. The hyperplasia developed on the 2d day and was a direct result of an intense cellular proliferation—the percentage of mitoses increased 5- to 15-fold. The character of this hyperplastic state differed completely from that produced by a similar continued local carcinogen treatment. Accordingly the hyperplasia-causing capacity of an EO derivative of Span 60, which contains, on the average, 20 EO units, *i.e.*, a product of Tween 60 character, is due to the compound itself. None of the EO derivatives of Span 20 caused either a high degree of or a persisting hyperplasia. Merely the addition of EO chains to Spans, in general, was not responsible for the effect, because the original Span type itself was decisive. This fact supports the significance of the physicochemical nature of the fatty acid ester-type, hyperplasia-causing agent.—J. Nat. Cancer Inst. 23: 953–967, 1959.

THE "PURE" tumor promotion process in mouse skin is fundamentally an entirely benign, intense proliferation of epidermal cells. As a result of this activity, a persisting typical hyperplasia evolves (*1–4*). When the development of hyperplasia is followed for a sufficiently long period (too short an observation period and/or too few skin biopsies will give an entirely misleading picture), it becomes evident that the tumor-promoting

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[3] Ethylene oxide derivatives of technical Span 60 and Span 20 were prepared by Atlas-Goldschmidt GMBH, Essen, Germany. We extend our thanks to Director H. Velde, Ph. D.

[4] Department of Chemistry, Institute of Technology.

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property and the hyperplasia-causing effect of dipole-type agents *always* have a positive correlation (*1–5*). This applies to all tumor-promoting agents we have studied. Of the tumor promoters of the dipole type, the polyol fatty acid ester of Tween 60 (polyoxyethylene sorbitan monostearate) is one of the most potent in both respects.

Ethylene oxide (*3, 5, 6*), when examined with a large number of different polyethylene glycols (Carbowaxes, Carbide and Carbon Chemical Co., New York, N.Y.) and Span 60—sorbitan monostearate (*3, 7*)—which are components of Tween 60, are ineffective. But some fatty acids that form the lipophilic portion of lipophilic-hydrophilic agents are themselves effective (*8, 9*). It is most likely that the hyperplastic effect of Tween 60 is due to the compound itself—chiefly because of its physicochemical nature. Thus the ability of the fatty acid ester-type agent to produce epidermal hyperplasia is significant (*1–7*).

The study reported here serves to substantiate the truth of the previously reported observations. In doing so, advantage is taken of the following observations: (1) Technical Tween 60 and laboratory-synthesized Tween 60 are equally effective (*4, 10, 11*). (2) Tween 60, which is obtained by adding ethylene oxide units to Span 60, is not a single compound but a mixture of closely related materials ("contaminating homologues") that contain a spectrum of ethylene oxide units with a distribution peak at the nominal value of about 20. The number of molecules of varying sizes is represented by Poisson's distribution formula (*cf, 12, 13*). It is to be expected, when various numbers of ethylene oxide units are added according to a predetermined plan to Span 60, that the resulting products will also be active over a wide range. (3) Span 60 is ineffective and its ethylene oxide derivative, Tween 60, is highly effective. Since Span 20 (sorbitan monolaurate) and its ethylene oxide derivative, Tween 20 (polyoxyethylene sorbitan monolaurate) possess a relatively weak and an extremely weak effect, respectively, the comparison of the effectiveness of Span 60 and Span 20 and their ethylene oxide derivatives is an applicable, methodical tool.

In the present study we have intentionally performed the experiments with technical products, which might contain more irrelevant by-products than the laboratory-synthesized compounds (*10, 14*).

MATERIALS AND METHODS

The methods used in these experiments have been described in the previous study (*4*). The animals were treated 6 times a week without preliminary treatment with carcinogen. The compounds used are shown in table 1. In order that the concentration of the compounds would be comparable with that of the preceding study (*4*), 0.18 M aqueous solutions and dispersions were used. Owing to the special nature of the agents, the value is approximate only.

Samples for electron microscopic examination were taken from all series

TABLE 1.—List of compounds used for treatment of skin on the back of mouse

Experiment No.	Synthesized Span 20	Technical Span	Nominal value of ethylene oxide (EO) units added	Concentration (*gm./100 ml.*)	Remarks
1		60	—	10.7	Sorbitan monostearate, but contains stearic acid and palmitinic acid.
2		60	5	14.7	
3		60	10	18.7	
4		60	15	22.7	
5		60	20	26.7	Should correspond to t. Tween 60.
6		60	30	34.8	
7		60	40	42.8	
8		60	60	58.9	
9		60	100	91.0	
10	20				
11		20	—	6.3	Sorbitan monolaurate, but contains lauric acid and small amounts of oleic, palmitinic, and stearic acid.
12		20	5	10.3	
13		20	10	14.3	
14		20	15	18.3	
15		20	20	22.3	Should correspond to t. Tween 20.
16		20	30	30.4	
17		20	40	38.4	
18		20	60	54.5	
19		20	100	86.6	
Synthesized Tween 60					Laboratory-synthesized polyoxyethylene sorbitan monostearate, containing 20 units EO (*10*).
Technical Tween 60					Polyoxyethylene sorbitan monostearate, but contains stearic acid and palmitinic acid.
Synthesized Tween 20					Laboratory-synthesized polyoxyethylene sorbitan monolaurate, containing 20 units EO (*10*).
Technical Tween 20					Polyoxyethylene sorbitan monolaurate, but contains lauric acid, and small amounts of oleic, palmitinic, and stearic acid.

(to be published later). In addition, a histologic control examination was made with laboratory-synthesized Span 20 (*10*).

In tables 2 and 3, data from another study (*4*) were used for the numerical values (previously unpublished) of the histoquantitative analyses. The cell populations in the interfollicular epidermis of the back of the mouse treated with s. and t. Tween 60 and 20 are shown in table 2. Table 3 shows the histoquantitative analyses after treatment with the carcinogens, 3-methylcholanthrene (MCA) and 7,12-dimethylbenz[*a*]anthracene (DMBA).

In the following tables, the standard error of the percentages of mitoses must be regarded as a rough mean only, in view of the small number of mitotic cells. All figures are percentages and ± = the S.E.

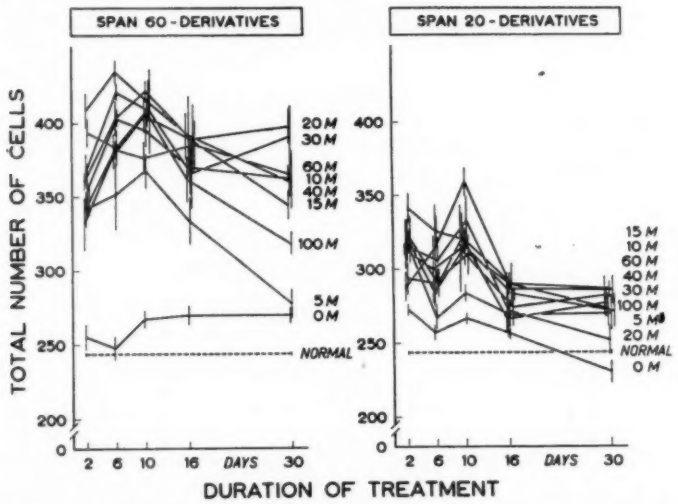
RESULTS

Normal Interfollicular Epidermis of the Back of the Mouse

The histologic structure of normal interfollicular epidermis (IFE) was described in detail in the preceding investigation. The distribution of the interfollicular epidermal-cell population in untreated animals is: Basal cells 71.1 ± 0.46, differentiating cells 25.4 ± 0.46, degenerating cells 3.48 ± 0.27, and mitotic cells 0.06 ± 0.02 (*4*).

Span 60 Ethylene Oxide Derivatives

The evolution and general development of hyperplasia in the IFE during a period of 30 days is shown in text-figure 1. Changes in the cell population are seen in table 4.



TEXT-FIGURE 1.—Development, degree, and evolution of interfollicular epidermal hyperplasia in the back of the mouse caused by Span 60 and Span 20 ethylene oxide derivatives.

TABLE 2.—Histoquantitative analyses of cell populations in the interfollicular epidermis of the back of the mouse treated with synthesized Tween 60 and 20 and technical Tween 60 and 20

Compound	Type of cells	Day							
		2		6		10		16		30	
s. Tween 60	Basal	54.7	± 1.40	56.3	± 1.00	58.5	± 1.70	54.5	± 1.30	56.0	± 1.30
	Differentiating	39.2	± 1.30	36.7	± 1.10	35.3	± 1.90	37.0	± 1.10	37.1	± 1.10
	Degenerating	4.8	± 0.58	6.7	± 0.85	5.7	± 0.40	7.5	± 0.42	6.4	± 0.56
	Mitotic	1.3	± 0.30	0.3	± 0.20	0.5	± 0.10	1.0	± 0.20	0.5	± 0.10
t. Tween 60	Basal	51.9	± 1.00	53.0	± 1.30	59.1	± 1.50	55.2	± 0.81	55.4	± 1.30
	Differentiating	41.5	± 1.30	38.8	± 1.00	35.0	± 0.72	36.2	± 1.10	36.4	± 0.74
	Degenerating	4.9	± 0.63	7.5	± 0.89	5.3	± 0.80	7.5	± 1.00	7.6	± 0.94
	Mitotic	1.7	± 0.20	0.7	± 0.30	0.6	± 0.30	1.0	± 0.20	0.6	± 0.20
s. Tween 20	Basal	58.4	± 0.70	57.7	± 2.00	59.0	± 1.00	62.4	± 0.50	57.6	± 1.80
	Differentiating	36.1	± 0.80	38.0	± 1.70	35.2	± 1.20	32.4	± 0.50	35.2	± 1.40
	Degenerating	4.6	± 0.47	3.2	± 0.60	5.3	± 0.54	4.6	± 0.81	6.2	± 0.36
	Mitotic	0.9	± 0.30	1.1	± 0.30	0.5	± 0.10	0.6	± 0.20	1.0	± 0.20
t. Tween 20	Basal	66.4	± 0.80	70.6	± 2.20	72.3	± 2.00	68.2	± 0.61	70.5	± 0.57
	Differentiating	31.2	± 0.42	27.8	± 2.20	25.7	± 1.70	30.4	± 0.58	27.5	± 0.75
	Degenerating	2.1	± 0.70	1.0	± 0.25	1.9	± 0.32	1.2	± 0.25	1.6	± 0.37
	Mitotic	0.3	± 0.10	0.6	± 0.20	0.1	± 0.10	0.2	± 0.20	0.4	± 0.20

TABLE 3.—Histoquantitative analyses of cell populations in the interfollicular epidermis of the back of the mouse treated with MCA and DMBA

Carcinogen	Dose (γ)	Type of cells	Day 2	Day 6	Day 10	Day 16	Day 30
MCA	0.06	Basal	74.2 ± 1.20	71.9 ± 2.10	72.5 ± 3.40	68.1 ± 1.50	67.7 ± 1.20
		Differentiating	24.9 ± 1.20	26.4 ± 2.10	25.4 ± 3.00	29.6 ± 1.00	29.4 ± 1.30
		Degenerating	0.7 ± 0.11	1.4 ± 0.52	2.0 ± 0.62	2.2 ± 0.58	2.5 ± 0.73
		Mitotic	0.2 ± 0.10	0.3 ± 0.10	0.1 ± 0.10	0.1 ± 0.10	0.4 ± 0.20
DMBA	0.06	Basal	74.7 ± 0.71	68.3 ± 1.10	66.1 ± 1.20	64.6 ± 2.30	66.4 ± 0.60
		Differentiating	24.2 ± 0.70	28.5 ± 0.90	30.5 ± 0.89	31.0 ± 2.10	29.8 ± 1.10
		Degenerating	1.0 ± 0.22	2.8 ± 0.35	3.3 ± 0.89	4.3 ± 0.89	3.4 ± 0.92
		Mitotic	0.1 ± 0.10	0.4 ± 0.10	0.1 ± 0.10	0.1 ± 0.10	0.4 ± 0.20
MCA	60	Basal	66.3 ± 1.90	65.5 ± 1.10	56.0 ± 1.70	61.8 ± 0.90	66.3 ± 0.50
		Differentiating	31.7 ± 2.20	30.9 ± 0.65	35.8 ± 1.90	32.3 ± 0.70	27.6 ± 0.52
		Degenerating	1.8 ± 0.47	3.1 ± 0.72	7.6 ± 0.40	5.8 ± 0.35	5.9 ± 0.47
		Mitotic	0.2 ± 0.10	0.5 ± 0.20	0.6 ± 0.10	0.1 ± 0.10	0.2 ± 0.20
DMBA	60	Basal	70.4 ± 1.30	72.1 ± 1.80	68.5 ± 1.70	62.1 ± 2.40	64.1 ± 1.90
		Differentiating	28.2 ± 1.10	25.6 ± 1.70	28.4 ± 1.50	34.3 ± 1.90	30.0 ± 2.40
		Degenerating	1.1 ± 0.52	1.8 ± 0.22	2.7 ± 0.22	3.3 ± 0.62	5.6 ± 0.93
		Mitotic	0.3 ± 0.20	0.5 ± 0.10	0.4 ± 0.20	0.3 ± 0.20	0.3 ± 0.10

TABLE 4.—*Histoquantitative analyses of cell populations in the interfollicular epidermis of the back of the mouse treated with technical Span 60 ethylene oxide derivatives*

Experiment No.	Nominal value of ethylene oxide units added to t. Span 60	Type of cells	Day 2	Day 6	Day 10	Day 16	Day 30
1	0	Basal	67.0 ± 0.72	65.4 ± 1.40	61.9 ± 0.72	58.1 ± 0.81	59.5 ± 1.10
		Differentiating	29.1 ± 0.90	30.6 ± 1.10	31.7 ± 0.97	34.1 ± 1.20	33.5 ± 0.78
		Degenerating	3.5 ± 0.33	3.2 ± 0.42	6.0 ± 1.00	7.8 ± 0.74	6.6 ± 0.92
		Mitotic	0.4 ± 0.10	0.8 ± 0.10	0.4 ± 0.20	0.1 ± 0.10	0.4 ± 0.30
2	5	Basal	67.0 ± 2.10	63.8 ± 1.20	64.3 ± 1.70	61.3 ± 0.92	63.7 ± 1.00
		Differentiating	29.2 ± 1.60	31.1 ± 1.10	31.2 ± 1.20	35.5 ± 0.62	33.2 ± 1.00
		Degenerating	3.2 ± 0.59	4.6 ± 0.40	3.8 ± 1.20	2.7 ± 0.27	2.5 ± 0.62
		Mitotic	0.5 ± 0.10	0.6 ± 0.30	0.7 ± 0.20	0.5 ± 0.10	0.6 ± 0.30
3	10	Basal	64.3 ± 1.40	66.8 ± 0.36	63.0 ± 2.20	68.8 ± 1.30	61.5 ± 1.60
		Differentiating	31.9 ± 1.40	29.5 ± 0.41	30.9 ± 1.50	26.6 ± 1.30	32.4 ± 1.40
		Degenerating	3.6 ± 0.40	3.2 ± 0.38	5.8 ± 0.75	4.3 ± 0.54	5.5 ± 0.70
		Mitotic	0.2 ± 0.10	0.5 ± 0.20	0.3 ± 0.10	0.3 ± 0.10	0.6 ± 0.30
4	15	Basal	69.2 ± 1.20	68.1 ± 1.10	65.3 ± 1.60	64.8 ± 0.65	63.7 ± 0.52
		Differentiating	27.4 ± 1.30	27.9 ± 1.00	30.3 ± 1.20	33.5 ± 1.00	31.7 ± 0.82
		Degenerating	3.0 ± 0.29	3.7 ± 0.38	3.9 ± 0.55	4.3 ± 0.56	4.1 ± 0.52
		Mitotic	0.4 ± 0.20	0.3 ± 0.20	0.5 ± 0.20	0.4 ± 0.20	0.5 ± 0.10
5	20	Basal	66.3 ± 1.20	64.9 ± 1.20	62.5 ± 1.30	63.0 ± 1.30	61.1 ± 1.30
		Differentiating	30.6 ± 1.10	29.7 ± 1.00	31.8 ± 1.10	30.8 ± 1.50	32.0 ± 1.50
		Degenerating	2.7 ± 0.42	5.0 ± 0.76	5.0 ± 0.70	5.9 ± 0.55	6.3 ± 1.00
		Mitotic	0.4 ± 0.10	0.4 ± 0.20	0.7 ± 0.20	0.3 ± 0.10	0.6 ± 0.10
6	30	Basal	65.0 ± 0.58	66.6 ± 2.20	66.9 ± 1.30	63.0 ± 1.00	67.6 ± 0.65
		Differentiating	31.2 ± 0.85	28.4 ± 1.70	28.3 ± 1.30	32.3 ± 1.20	26.9 ± 1.00
		Degenerating	3.0 ± 0.63	4.7 ± 0.77	4.4 ± 0.83	4.4 ± 0.37	5.1 ± 1.00
		Mitotic	0.8 ± 0.20	0.3 ± 0.10	0.4 ± 0.10	0.3 ± 0.10	0.4 ± 0.20
7	40	Basal	67.8 ± 1.00	65.4 ± 0.97	70.2 ± 1.00	62.2 ± 1.50	61.9 ± 1.10
		Differentiating	28.0 ± 0.88	27.9 ± 0.62	24.9 ± 1.10	32.7 ± 1.80	31.9 ± 0.79
		Degenerating	3.9 ± 0.62	6.4 ± 0.50	4.5 ± 0.37	4.3 ± 0.72	5.3 ± 0.52
		Mitotic	0.3 ± 0.10	0.3 ± 0.10	0.4 ± 0.20	0.8 ± 0.20	0.9 ± 0.40
8	60	Basal	63.1 ± 0.90	65.5 ± 1.00	64.2 ± 0.47	64.4 ± 1.50	60.2 ± 2.00
		Differentiating	33.1 ± 0.81	29.2 ± 1.40	29.6 ± 1.30	30.8 ± 1.30	33.7 ± 1.50
		Degenerating	3.2 ± 0.76	4.7 ± 0.91	5.6 ± 0.96	4.6 ± 0.41	5.2 ± 0.52
		Mitotic	0.6 ± 0.20	0.6 ± 0.30	0.6 ± 0.10	0.2 ± 0.10	0.9 ± 0.20
9	100	Basal	68.8 ± 1.50	63.9 ± 2.50	62.1 ± 1.20	60.8 ± 1.20	57.6 ± 1.50
		Differentiating	28.8 ± 1.50	30.9 ± 2.40	30.3 ± 1.00	33.1 ± 0.85	36.3 ± 1.30
		Degenerating	2.0 ± 0.69	4.4 ± 0.43	6.4 ± 0.81	5.5 ± 0.47	5.3 ± 0.67
		Mitotic	0.4 ± 0.20	0.8 ± 0.10	1.2 ± 0.20	0.6 ± 0.10	0.8 ± 0.30

Experiment 1.—Histologic changes in the IFE of the back of the mouse were the same as with s. Span 60 (*4*). On the 2d day the IFE was somewhat thicker than normal, while its cells were slightly above normal in size. There were some mitotic figures. On the 6th and 10th days the stratum granulosum and the stratum corneum were slightly thickened. On the 16th and 30th days the alterations were still of the same kind and degree; here and there the IFE again appeared normal (fig. 1).

Experiment 2.—On the 2d day the cells of IFE had clearly increased in size. The cells formed 2 or 3 layers; intercellular spaces had developed and were of considerable width in some places. At the same time the intercellular bridges were well differentiated. Some cells exhibited intracellular edema and perinuclear vacuolation. On the 6th day interfollicular epidermal hyperplasia was pronounced. The cells were in 3 or 4 layers. On the 10th day the situation was unchanged, but on the 16th day there were fewer alterations and on the 30th day there was hardly any hyperplasia, though the total cell number was somewhat above normal.

Experiment 3.—On the 2d day the interfollicular epidermal hyperplasia was pronounced. Cells were in 3 or more layers. Intercellular spaces were wide throughout and the intercellular bridges were well differentiated. Numerous cells exhibited intracellular edema and perinuclear vacuolation. On the 6th day the degree of hyperplasia was still greater. Parakeratosis was a usual occurrence. After the 6th day the alterations remained similar; intracellular edema persisted but the degree of vacuolation had decreased. The stratum granulosum and the stratum corneum had thickened evenly. Neither cellular nor nuclear atypia had developed and there were no disturbances in organization.

Experiments 4 through 8.—This series showed hyperplastic alterations that were the same as those found in experiment 3. No cellular or nuclear atypia, nor any disturbances in organization, occurred during the entire observation period (figs. 2 and 3). The nature and degree of hyperplasia was the same as that produced by treatment with t. and s. Tween 60 (*4*).

Experiment 9.—Alterations in IFE on the 2d, 6th, and 10th days were of the same degree and nature as in experiments 3 through 8, in which an average of 10 to 60 units of ethylene oxide was added to t. Span 60. However, from the 16th day there was a definite difference; from this time the degree of alterations became less. This was especially evident on the 30th day; the cells of the stratum malpighii were slightly smaller than those on the earlier observation days.

Epilation did not occur in any of the mice. Those agents that produced the most pronounced hyperplasia in the IFE also produced hyperplastic alterations and some keratinization in the openings of hair follicles. With the exception of experiment 1, the mice in all other experiments exhibited edema and inflammatory reaction in the dermis.

Histoquantitative analyses (text-fig. 1 and table 4) revealed that t. Span 60 caused no noteworthy increase in the total number of cells during

the entire treatment period. In experiments 2 through 9, in which the compounds contained various amounts of ethylene oxide units, the total cell number had markedly increased by the 2d day. The total number of cells was highest on the 6th and 10th days. Thereafter, the alterations produced by t. Span 60 + 5 EO and t. Span 60 + 100 EO differed from those in experiments 3 through 8. These differences were significant on the 30th day. In experiments 4 through 8, analyses further showed that the total cell number as well as the cell population distributions were close, *i.e.*, when the nominal number of ethylene oxide units of the compounds was from 10 to 60. In this range of the compound spectrum, the percentage of differentiating cells had clearly increased but that of the basal cells was practically unchanged. These values correspond to those (table 2) induced with s. or t. Tween 60, in which the nominal value of ethylene oxide units is about 20. All of these values differed from those obtained in the experiments in which the mice were treated with the carcinogens MCA and DMBA [table 3; *see* text-fig. 2 in (*4*)].

Span 20 Ethylene Oxide Derivatives

The general course of the alterations in IFE is seen in text-figure 1. Changes in cell population are shown in table 5.

Experiments 10 and 11.—Aqueous dispersions of Span 20 used in this study contained only 6.3 gm. of substance in 100 ml. of solution. In our earlier experiments t. Span 20 was used in the anhydrous state (*5*). S. and t. Span 20 did not produce cellular or nuclear atypia, or disturbances in organization (*cf. 2, 3*). It first appeared that changes produced in IFE by the two agents were similar. On the 2d day the IFE had slightly thickened, and the cells were larger than normal. Intercellular spaces were discernible in many areas and the cells usually were in 2 layers. On the 6th, 10th, and 16th days the stratum granulosum and the stratum corneum were thicker than normal. On the 30th day, the cells of IFE were practically normal in size, but the stratum granulosum and the stratum corneum remained thickened (fig. 4).

Experiments 12 through 19.—Histologic alterations in IFE were almost the same in these 8 experiments. It was a general feature that on the 2d day the cells had, comparatively, clearly increased in size. Intercellular spaces were distinguishable, though not markedly. The total cell number had increased in all experiments, but was lower than in the corresponding Span 60 experiments. Cells were in 2 or at most 3 layers. Indicative of a weak effect were the variations, on the 6th day, in the intensity of the interfollicular epidermal reaction in different animals of the same experiments—in some animals the IFE was definitely hyperplastic, in others nearly normal. On the 10th day the degree of hyperplasia was possibly highest, but on the 16th day the reaction had definitely decreased or was decreasing in all experiments. Marked hyperplasia was noted in only a few animals. Intercellular spaces were disappearing and by the 30th day the thickness of the IFE had greatly

TABLE 5.—Histoquantitative analyses of cell populations in the interfollicular epidermis of the back of the mouse treated with technical Span 20 ethylene oxide derivatives

Experiment No.	Nominal value of ethylene oxide units added to t. Span 20	Type of cells	Day 2	Day 6	Day 10	Day 16	Day 30
11	0	Basal	67.1 ± 0.54	67.6 ± 1.10	69.4 ± 1.10	66.8 ± 0.82	74.1 ± 0.64
		Differentiating	31.5 ± 0.85	30.1 ± 0.58	28.2 ± 1.60	31.1 ± 1.30	24.3 ± 1.10
		Degenerating	1.1 ± 0.43	2.0 ± 0.69	1.5 ± 0.40	1.6 ± 0.35	1.3 ± 0.27
		Mitotic	0.3 ± 0.10	0.3 ± 0.10	0.9 ± 0.20	0.5 ± 0.20	0.3 ± 0.30
12	5	Basal	71.5 ± 1.40	65.8 ± 1.60	67.0 ± 1.60	66.5 ± 0.84	66.2 ± 1.00
		Differentiating	25.9 ± 1.50	30.6 ± 1.40	30.1 ± 1.10	30.9 ± 0.80	30.0 ± 0.81
		Degenerating	1.9 ± 0.44	3.0 ± 0.35	2.4 ± 0.57	2.2 ± 0.27	3.0 ± 0.94
		Mitotic	0.7 ± 0.20	0.6 ± 0.20	0.5 ± 0.20	0.4 ± 0.20	0.8 ± 0.10
13	10	Basal	65.5 ± 1.20	67.0 ± 1.50	65.8 ± 1.90	67.0 ± 1.90	62.9 ± 1.30
		Differentiating	31.0 ± 1.40	30.6 ± 1.90	30.7 ± 1.80	30.4 ± 1.60	33.9 ± 1.10
		Degenerating	3.1 ± 0.42	2.1 ± 0.49	3.2 ± 0.67	2.1 ± 0.57	2.8 ± 0.52
		Mitotic	0.4 ± 0.20	0.3 ± 0.20	0.3 ± 0.10	0.5 ± 0.20	0.4 ± 0.10
14	15	Basal	65.8 ± 1.50	63.8 ± 0.95	64.6 ± 0.68	64.8 ± 1.10	62.8 ± 0.82
		Differentiating	30.0 ± 0.86	31.3 ± 1.40	30.5 ± 0.93	30.9 ± 0.97	33.2 ± 0.78
		Degenerating	3.5 ± 0.54	4.6 ± 0.56	4.7 ± 0.76	3.8 ± 0.43	3.5 ± 0.53
		Mitotic	0.7 ± 0.20	0.3 ± 0.20	0.2 ± 0.10	0.5 ± 0.20	0.5 ± 0.20
15	20	Basal	63.4 ± 2.40	65.2 ± 2.10	64.7 ± 2.10	65.1 ± 2.80	65.3 ± 1.50
		Differentiating	33.3 ± 2.00	30.0 ± 1.60	29.6 ± 1.60	30.3 ± 2.20	30.8 ± 1.60
		Degenerating	2.2 ± 0.45	4.2 ± 0.72	5.1 ± 1.20	4.4 ± 0.82	3.2 ± 0.76
		Mitotic	1.1 ± 0.30	0.3 ± 0.10	0.6 ± 0.20	0.2 ± 0.10	0.7 ± 0.20
16	30	Basal	69.3 ± 1.70	64.1 ± 1.00	66.2 ± 1.30	67.3 ± 1.30	63.5 ± 0.33
		Differentiating	28.4 ± 1.20	33.3 ± 1.40	30.3 ± 0.70	29.2 ± 0.79	31.9 ± 0.58
		Degenerating	1.9 ± 0.29	2.4 ± 0.70	2.9 ± 0.65	3.4 ± 1.10	4.0 ± 0.43
		Mitotic	0.4 ± 0.20	0.2 ± 0.10	0.6 ± 0.10	0.1 ± 0.10	0.6 ± 0.20
17	40	Basal	68.8 ± 1.70	64.0 ± 0.72	64.3 ± 1.60	68.4 ± 1.30	60.7 ± 1.20
		Differentiating	27.6 ± 1.30	31.9 ± 0.76	30.1 ± 1.30	28.8 ± 1.10	32.6 ± 1.10
		Degenerating	3.1 ± 0.63	3.8 ± 0.16	5.0 ± 1.30	2.7 ± 0.31	6.1 ± 0.83
		Mitotic	0.5 ± 0.10	0.3 ± 0.10	0.6 ± 0.10	0.1 ± 0.10	0.6 ± 0.20
18	60	Basal	64.4 ± 1.40	62.6 ± 1.00	62.6 ± 1.30	66.9 ± 1.30	59.9 ± 1.70
		Differentiating	31.3 ± 1.30	34.0 ± 1.10	32.6 ± 0.63	30.2 ± 1.30	34.7 ± 1.60
		Degenerating	3.5 ± 0.27	3.3 ± 0.61	4.5 ± 1.10	2.6 ± 0.40	5.0 ± 1.10
		Mitotic	0.8 ± 0.30	0.1 ± 0.10	0.3 ± 0.20	0.3 ± 0.10	0.4 ± 0.20
19	100	Basal	68.4 ± 1.80	63.4 ± 2.70	64.5 ± 1.50	65.1 ± 0.58	60.5 ± 1.60
		Differentiating	28.8 ± 1.60	32.2 ± 1.80	31.0 ± 0.87	31.3 ± 0.86	33.0 ± 1.00
		Degenerating	2.4 ± 0.72	3.8 ± 0.82	4.2 ± 1.10	3.5 ± 0.54	6.2 ± 1.30
		Mitotic	0.4 ± 0.20	0.6 ± 0.30	0.3 ± 0.10	0.1 ± 0.10	0.3 ± 0.20

diminished in all experiments. The stratum granulosum and the stratum corneum were thicker than normal throughout the experimental period. No cellular or nuclear atypia or disturbances in organization were present (figs. 5 and 6). The nature and degree of the interfollicular epidermal hyperplasia was the same as that which developed as a result of treatment with s. and t. Tween 20 (*4*).

No noteworthy follicular changes occurred in any of the experiments. The thickness of the dermis was slightly above normal and a scanty number of inflammatory cells was seen.

Histoquantitative analyses (text-fig. 1 and table 5) in the experiments with s. and t. Span 20 revealed morphologic characteristics related to those in experiments 12 through 19. The increase in the total cell number was essentially due to an increase in the differentiating cells. At the same time all these values differed from those in the experiments with carcinogens (table 3; *see also* table 2).

DISCUSSION

We have previously demonstrated that technical Tween 60 and laboratory-synthesized Tween 60, which was obtained by adding about 20 units of ethylene oxide to laboratory-synthesized Span 60, were equally efficient as tumor promoters for mouse skin (*11*). In addition, they have a pronounced, persisting hyperplasia-causing effect (*4*).

In the first part of this investigation (expts. 1 through 9) a study was made of the epidermal hyperplasia produced in the IFE of the back of the mouse by technical Span 60 and its derivatives of the Tween 60 type which contained various numbers of ethylene oxide units. It was found that all products of the Tween 60 type caused a high degree of regular interfollicular epidermal hyperplasia. Furthermore, the evolution, general development, and nature of this condition were the same as those caused by technical Tween 60 and by the laboratory-synthesized Tween 60. The hyperplasia-causing effect, moreover, was practically independent of the mole ratio distribution in the individual compounds—only the hyperplasia caused by products containing 5 and 100 ethylene oxide units regressed with time. The hyperplastic response of the IFE caused by these products was quite different from and opposite to that of the locally applied carcinogens, MCA and DMBA [*see especially* (*1–4*)].

Although compounds of the Tween 60 type [5] have this property, it does not mean that *all* other *Tweens* would also have the same property. On the contrary, there are also almost inactive Tweens (*1*, *3*, *5*).

In earlier experiments we have shown that technical and synthesized Tween 20, which was obtained by the addition of ethylene oxide from laboratory-synthesized Span 20, are almost ineffective either as tumor

[5] Tween 40, polyoxyethylene sorbitan monopalmitate, is nearly as effective as a tumor promoter (*3*, *5*, *7*) and hyperplasia-causing agent as Tween 60 (*1–5*).

promoters or as hyperplasia-causing agents (*4*, *11*). There was no improvement of the effect when they were applied twice a day.

In experiments 10 through 19 the addition of ethylene oxide chains, in any ratio, to technical Span 20 resulted in a Tween 20-type compound that did not possess the property of producing a high-degree persisting hyperplasia. Our earlier observations were confirmed (*15*). Regardless of the continued application of these derivatives, the interfollicular epidermal hyperplasia gradually regressed.

The observations made in the present study plus the evidence of our previous work seem to indicate the following: (1) The epidermal hyperplasia-causing effect of the polyol fatty acid ester of the Tween 60-type compound depends on the ester itself—chiefly, on its physicochemical nature. Merely the addition of ethylene oxide chains to Spans in general is not responsible for the effect, as the Span 60 type is itself decisive. (2) The hyperplasia is a result of greatly intensified, continued proliferation of cells (the percentage of mitoses increased from 5- to 15-fold and the percentage of differentiating cells increased significantly). The hyperplasia is not a result of a delayed maturation of the epidermal cells (3). The evolution, general development, and nature of this hyperplasia differs completely in all essential respects from the hyperplastic condition caused by the local and continued application of 0.06 and 60 γ of 3-methylcholanthrene and 0.06 and 60 γ of 7,12-dimethylbenz[*a*]anthracene.

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PLATES

PLATE 90

Photomicrographs taken on 30th day: Hemalum and eosin. × 530

FIGURE 1.—Mouse #7408. *T. Span 60.* IFE slightly thicker than normal; cells nearly normal in size, shape, and number.

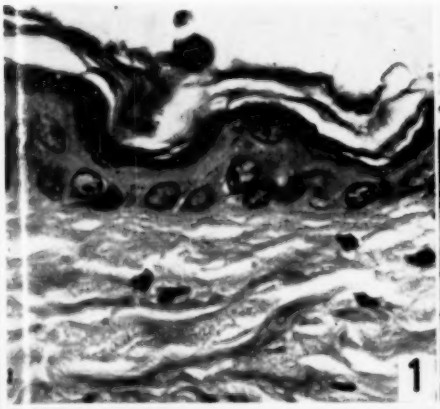
FIGURE 2.—Mouse #7560. *T. Span 60 + 20 EO* (corresponds to *t. Tween 60*). High-degree IFE hyperplasia. Large cells and nuclei; intercellular spaces wide and intercellular bridges well differentiated. Stratum granulosum rather thick, corresponding to degree of hyperplasia; stratum corneum detached.

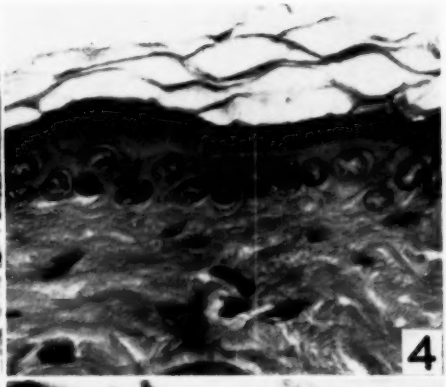
FIGURE 3.—Mouse #7635. *T. Span 60 + 60 EO.* High-degree, regular IFE hyperplasia similar to that in figure 2. Considerable parakeratosis. (An alteration associated with intercellular edema.)

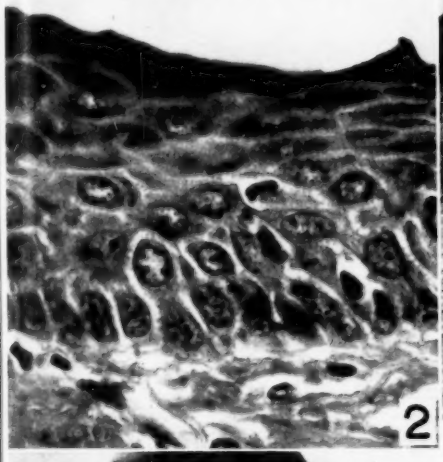
FIGURE 4.—Mouse #9285. *T. Span 20.* Only slightly thickened IFE; number of cells somewhat increased and fairly normal in size.

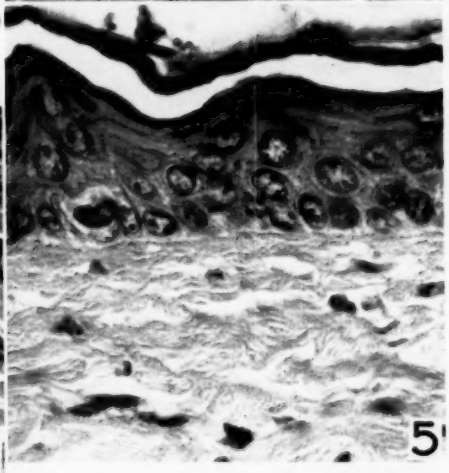
FIGURE 5.—Mouse #7760. *T. Span 20 + 20 EO* (corresponds to *t. Tween 20*). Low-degree IFE hyperplasia. Cells larger than normal. Narrow intercellular spaces discerned. (*Cf.* fig. 2, which shows a high-degree IFE hyperplasia.)

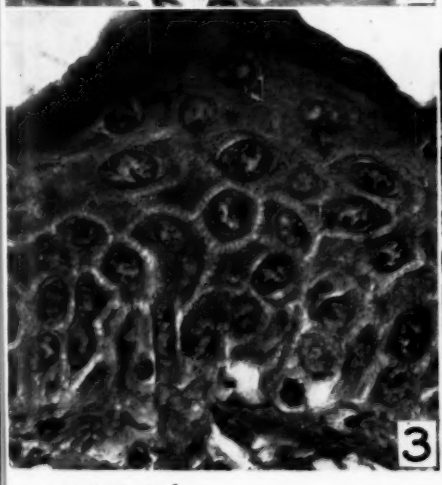
FIGURE 6.—Mouse #7831. *T. Span 20 + 60 EO.* IFE the same as in figure 5. Cells only slightly larger, while the number has increased. (*Cf.* fig. 3, which shows a high-degree IFE hyperplasia.)

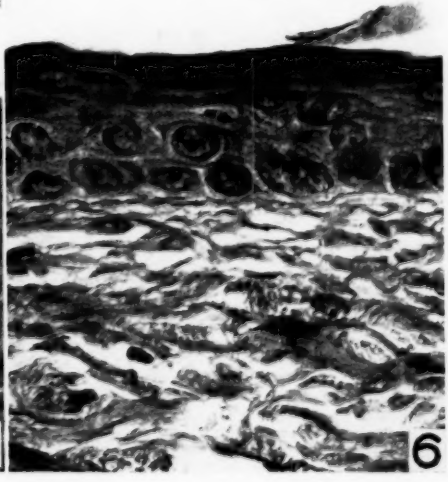












523596—59——11

Mechanism of Experimental Tumorigenesis. III. Effect in Mouse Skin of 1,4-Sorbitan and 1,4,3,6-Dianhydrosorbitol Stearates[1,2]

KAI SETÄLÄ, LAURI MERENMIES, LEO STJERNVALL, YRJÖ AHO,[3] *and* PAAVO KAJANNE,[3] *Department of Pathology, University of Helsinki, and Department of Chemistry, Institute of Technology, Helsinki, Finland*

SUMMARY

When 1,4-sorbitan and 1,4,3,6-dianhydrosorbitol were substituted for the sugar alcohol in Span 60 (sorbitan monostearate) and Tween 60 (polyoxyethylene sorbitan monostearate), and the resulting products ≈0.18 M aqueous dispersions and solutions were painted 6 times a week on the mouse skin, the alterations in the skin of the back of the mouse corresponded to those caused by the original Span 60 and Tween 60, respectively. The Span 60-type products 1,4-sorbitan monostearate, 1,4-sorbitan distearate, 1,4-sorbitan tristearate, 1,4,3,6-dianhydrosorbitol monostearate, and 1,4,3,6-dianhydrosorbitol distearate were almost inactive. The Tween 60-type products polyoxyethylene-1,4-sorbitan monostearate, polyoxyethylene-1,4-sorbitan distearate, polyoxyethylene-1,4-sorbitan tristerate, and polyoxyethylene-1,4,3,6-dianhydrosorbitol monostearate produced an immediately developing, high-degree, and regular epidermal hyperplasia. The evolution, general development, and nature of this hyperplasia were similar in all respects to those produced by Tween 60. This is one fact in favor of the significance of the physicochemical nature of a fatty acid ester-type agent which causes hyperplasia.—J. Nat. Cancer Inst. 23: 969–977, 1959.

IT HAS been adequately demonstrated that the property of Tween 60 (polyoxyethylene sorbitan monostearate), a compound of dipole nature which promotes tumors and causes hyperplasia, is due to its physicochemical character (*1*, *2*). Technical products and laboratory-synthesized compounds are equally potent (*3*, *4*),

If, in the production of new Tween 60-type products, the synthesis is interrupted at the Span 60 (sorbitan monostearate) stage, the resulting products are biologically inactive. But if the synthesis is continued by the addition of ethylene oxide units to Tween 60, highly active compounds result (*1–5*). The activity is not essentially dependent on the mole ratio distribution of ethylene oxide (*2*, *6*).

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[3] Department of Chemistry, Institute of Technology, Helsinki, Finland.

523596—59——12

Using as "models" the structure of Span 60 and Tween 60, respectively, we have, on the basis of the preceding principle, prepared numerous new products. The important observation was made that other alcohols may well be substituted for the sugar alcohol contained in Spans and Tweens (*4*, *5*). In the first stage of this line, monoanhydride (1,4-sorbitol) and dianhydride (1,4,3,6-dianhydrosorbitol) were substituted for the sugar alcohol. The present paper describes the histologic alterations in the epidermis that were produced by treatment of the skin of the back of the mouse with 1,4-sorbitol and 1,4,3,6-dianhydrosorbitol stearates of Span 60 and Tween 60 type.

MATERIALS AND METHODS

The general experimental procedures were similar to those which were used in our previous studies in this series (*1*, *2*).

The backs of the mice were painted once daily (6 times a week, Sundays excepted), without preceding carcinogen application, with the following polyol fatty acid esters (*see* text-fig. 1 for the structural formulas of these products) synthesized by us (*4*): The 1,4-sorbitan ("arlitan") products: (1) 1,4-sorbitan monostearate, (2) 1,4-sorbitan distearate, (3) 1,4-sorbitan tristearate and their ethylene oxide derivatives (containing an average of 20 units of ethylene oxide), (4) polyoxyethylene-1,4-sorbitan monostearate, (5) polyoxyethylene-1,4-sorbitan distearate, and (6) polyoxyethylene-1,4-sorbitan tristearate; the 1,4,3,6-dianhydrosorbitol ("isosorbide") products: (7) 1,4,3,6-dianhydrosorbitol monostearate, (8) 1,4,3,6-dianhydrosorbitol distearate, and (9) the ethylene oxide derivative polyoxyethylene-1,4,3,6-dianhydrosorbitol monostearate (with about 20 ethylene oxide units). Additionally we studied the effect of a number of intermediate crystallization products.

All the compounds were used in the concentration of ≈ 0.18 M as previously (*1*, *2*). Since compounds #1 to 3, 7 and 8, which here are termed "Span 60" type, are insoluble in water, they were used as fine dispersions in 100 percent Carbowax 400.[4] Compounds #4 to 6, and 9, again, which are termed "Tween 60" type, are water soluble.

At the same time that the biopsy specimens were taken for light microscopic examination, samples were also taken and prepared for electron microscopic examination (to be published later).

We have previously demonstrated that tumor promoters of dipole nature produce, by the 2d and the 6th days, the epidermal changes characteristic of them, but the actual carcinogens produce them later (*1*, *2*, *7–9*). Specific attention is therefore drawn in this report to this "critical period."

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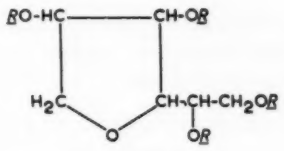
[4] Polyethylene glycol 400, average molecular weight 380 to 420, completely soluble in water (Carbide and Carbon Chemical Co., New York, N.Y.).

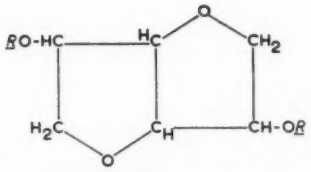


1,4-sorbitan (arlitan) 4 R = H;
1,4-sorbitan-monostearate 3 R = H; 1 R = Stearoyl group
1,4-sorbitan-distearate 2 R = H; 2 R = Stearoyl group
1,4-sorbitan-tristearate 1 R = H; 3 R = Stearoyl group

Polyoxyethylene-1,4-sorbitan-monostearate 3 R = POE; 1 R = Stearoyl group
Polyoxyethylene-1,4-sorbitan-distearate 2 R = POE; 2 R = Stearoyl group
Polyoxyethylene-1,4-sorbitan-tristearate 1 R = POE; 3 R = Stearoyl group

Stearoyl group: $C_{17}H_{35}CO$

Polyoxyethylene (POE) group: $-(CH_2-CH_2-O)_n-H$



1,4,3,6-dianhydrosorbitol (isosorbide) 2 R = H;
1,4,3,6-dianhydrosorbitol-monostearate 1 R = H; 1 R = Stearoyl group
1,4,3,6-dianhydrosorbitol-distearate 2 R = Stearoyl group

Polyoxyethylene-1,4,3,6-dianhydrosorbitol-monostearate 1 R = POE; 1 R = Stearoyl group

Stearoyl group = $C_{17}H_{35}CO$
Polyoxyethylene (POE) group = $-(CH_2-CH_2-O)_n-H$

TEXT-FIGURE 1.—Schematic drawings of 1,4-sorbitan and 1,4,3,6-dianhydrosorbitol stearates synthesized by us (*4*).

RESULTS

Solvent Control

Continuous treatment with undiluted Carbowax 400 produced in the interfollicular epidermis (IFE) only a slight increase in cell number on the back of the mouse. This resulted in a thickening of the IFE on the 6th day (fig. 1). Hyperplasia did not develop, and no cellular or nuclear atypia or disturbances in organization were seen. The pilosebaceous

apparatus was intact, with no epilation. In numerous specimens the IFE cell boundaries were indistinct, while the dermis exhibited slight edematous and inflammatory changes.

1,4-Sorbitan

Experiment 1 (*1,4-sorbitan monostearate*).—The light microscopic alterations were insignificant. The IFE was somewhat thicker than normal and the cells were probably larger than normal, but there was no cellular or nuclear atypia. In appearance, the IFE was approximately similar to that after treatment with Carbowax 400 alone. No intercellular spaces were present (fig. 2).

Experiments 2 (*1,4-sorbitan distearate*) and *3* (*1,4-sorbitan tristearate*).—In principle, the light microscopic appearance of the IFE was similar to that in experiment 1. No differences were seen between these two experiments.

Experiment 4 (*polyoxyethylene-1,4-sorbitan monostearate*).—In contrast to the preceding experiments, IFE hyperplasia now developed. On the 2d day the cells of the IFE were larger than normal and were in 2 layers. On the 6th day intercellular spaces were seen here and there, and in addition, a mild intracellular edema had developed. Intercellular bridges were well differentiated with the cells chiefly situated in 2 or 3 regular layers. There was no cellular or nuclear atypia or disturbances in organization. The stratum granulosum and stratum corneum were above normal in thickness and no epilation occurred.

Experiment 5 (*polyoxyethylene-1,4-sorbitan distearate*).—IFE alterations were the same in principle as in experiment 4, but they were clearly different in degree. The degree of hyperplasia was definitely greater on the 2d day. The cells were large and showed intracellular edema. Intercellular spaces were wide with intercellular bridges well differentiated and stratum granulosum thicker than normal. On the 6th day (fig. 4) the situation was practically unchanged: The cells of stratum malpighii were usually in 3 layers and stratum granulosum and stratum corneum were thicker than normal. The pilosebaceous apparatus was everywhere intact, with no epilation. The dermis exhibited mild edema and inflammatory reaction.

Experiment 6 (*polyoxyethylene-1,4-sorbitan tristearate*).—This treatment caused a higher degree of IFE hyperplasia than the corresponding mono- and distearates (expts. 4 and 5), and this high-degree hyperplasia was present immediately. On the 2d day, the cells, which were markedly large, were situated in 3 layers, with many cells showing intracellular edema. Intercellular spaces were very wide and were seen everywhere, and there was definite parakeratosis. On the 6th day (fig. 6) the degree of hyperplasia was further increased. The cells of stratum malpighii were large and in 3 to 4 layers. No cellular or nuclear atypia or disturbances in organization were seen. The stratum granulosum and stratum corneum were thick. The pilosebaceous apparatus remained

intact, with no epilation. The dermis showed mild edema and inflammatory reaction.

1,4,3,6-Dianhydrosorbitol

Experiment 7 (*1,4,3,6-dianhydrosorbitol monostearate*).—No IFE hyperplasia developed at any time during the experimental period. The alterations were similar in degree and nature to those in the 1,4-sorbitan monostearate series (expt. 1). On the 2d day the cells in 2 layers were possibly somewhat increased in size, with occasional narrow intercellular spaces. On the 6th day (fig. 3) the changes were exactly as before: The stratum granulosum and stratum corneum were somewhat thicker than normal and alterations in the dermis were extremely slight.

Experiment 8 (*1,4,3,6-dianhydrosorbitol distearate*).—The effect of this compound differed from that of all other compounds not containing ethylene oxide units (expts. 1 to 3, and 7) in that mild IFE hyperplasia developed in some animals. In addition, in some parts of the treated area of even the same animal there was no hyperplasia. Figure 5 shows the highest degree of IFE hyperplasia on the 6th day. Portions of the treated area on this animal had no hyperplastic alterations. There was no cellular or nuclear atypia or disturbances in organization.

Experiment 9 (*polyoxyethylene-1,4,3,6-dianhydrosorbitol monostearate*).—A relatively high-degree IFE hyperplasia developed immediately. On the 2d day the cells, in 2 to 3 layers, were greatly increased in size, and in many areas they showed intracellular edema, with intercellular spaces. On the 6th day (fig. 7) the degree of hyperplasia was higher than on the 2d day. Cells in 3 to 4 layers were large and separated by wide intercellular spaces, with intercellular bridges well differentiated. There was no cellular or nuclear atypia or disturbances in organization, but marked parakeratosis was present here and there. The pilosebaceous apparatus remained normal, with no epilation. Dermis was somewhat edematous and contained a few inflammatory cells.

DISCUSSION

It was demonstrated that of the new products synthesized according to the "models" of Span 60 and Tween 60, by substituting 1,4-sorbitan and 1,4,3,6-anhydrosorbitol for sugar alcohol, products of Span 60 type applied to the mouse skin (6 times a week) were ineffective. On the contrary, as was to be expected (*e.g., 1,2,5,6*), their ethylene oxide derivatives of Tween 60 type produced an immediately developing, high-degree and regular ("tissue preserving") epidermal hyperplasia and an increase in the amount of cells in differentiation (*see* also *1, 2, 6–9*). Briefly stated, there developed an essentially similar IFE hyperplasia which was demonstrated to be characteristic of all the tumor-promoting substances of the

dipole type studied so far (*1, 3, 7–10*). Furthermore, the evolution, general development, and nature of the hyperplasia were, as before, largely opposite to the epidermal condition produced by the carcinogen itself, as paradoxical as this may seem (*cf. 7–9*).

With our experimental observations made earlier and with those now made, it is justified to present the following chain of evidence: The laboratory-synthesized compounds used in the present study contained the esters of only one fatty acid (stearic acid), which was also true of the pure Tween 60 earlier synthesized (*1, 3, 4*). All these ethylene oxide derivatives are equally effective as agents causing epidermal hyperplasia as the corresponding original "model" (technical Tween 60). It is also known that Tween 40 (polyoxyethelene sorbitan monopalmitate) is as effective a tumor promoter (*7, 11*) and an agent which causes hyperplasia as those derivatives of Tween 60 type, but Tween 20 (polyoxyethylene sorbitan monolaurate) has a very weak potency (*9, 11*). Furthermore, certain pure fatty acids that form the lipophilic portion of these lipophilic-hydrophilic agents are effective tumor promoters (*10*) and agents causing hyperplasia (*1*).

Finally, when we remember that sugar alcohol in Tween 60-type compounds may also be replaced by other alcohols, especially glycerol (*5*), without alteration of the biological effect, we have at our disposal substances that are physiologic and, furthermore, in the same form as lipides in the living organism (glycerol, as we know, is the parent substance of natural fats, oils, and phosphatides). Accordingly, direct experimental data have again been demonstrated in favor of the significance of the physicochemical nature of fatty acid ester-type agents which cause epidermal hyperplasia in mice.

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PLATE 91

Microphotographs taken on the 6th day. Hemalum and eosin. × 530

FIGURE 1.—Mouse #8957. Carbowax (solvent control). IFE somewhat thicker than normal; cell boundaries indistinct.

FIGURE 2.—Mouse #8961. 1,4-sorbitan monostearate. IFE slightly thicker than normal; cell margins indistinct.

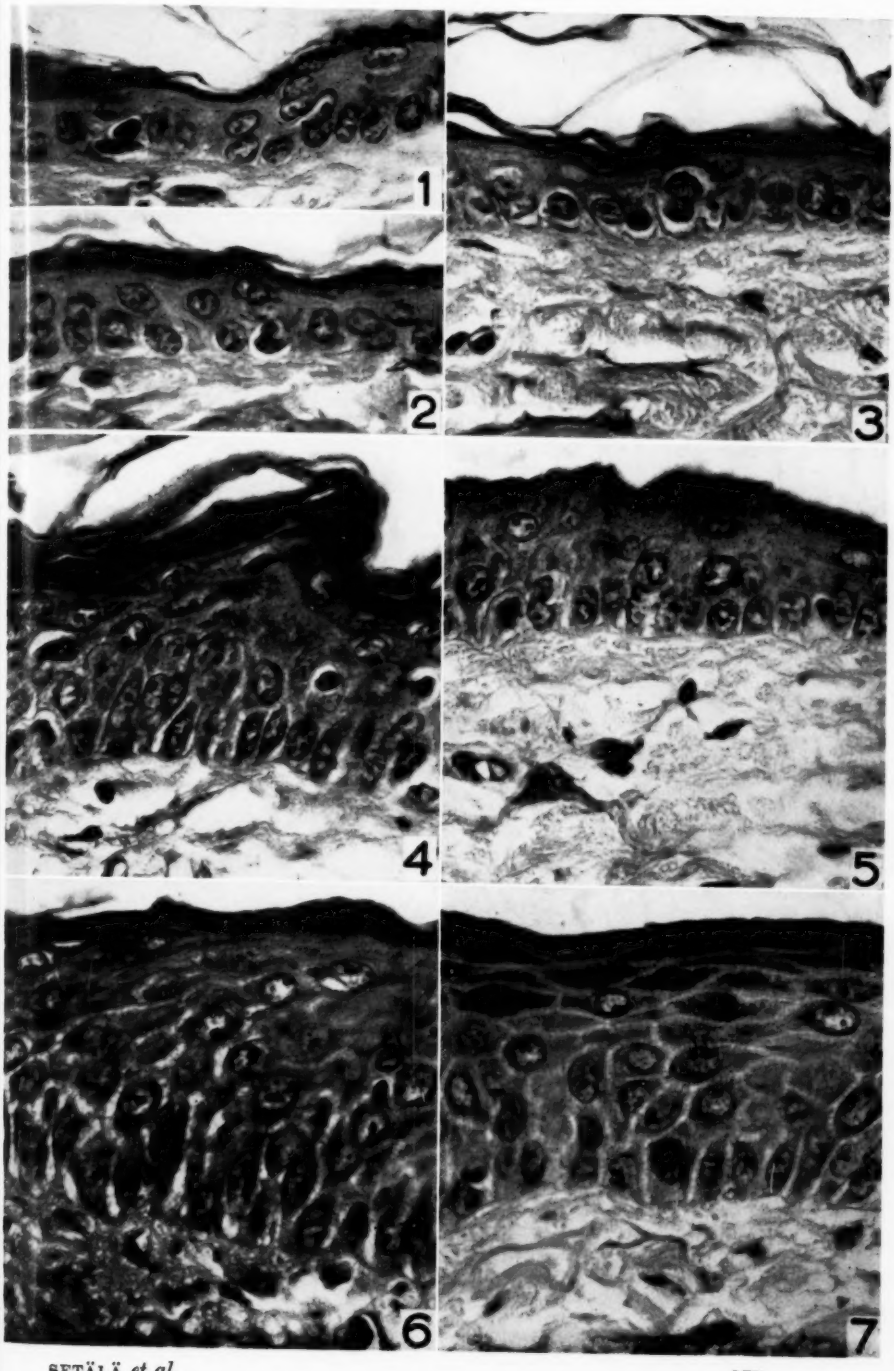
FIGURE 3.—Mouse #8987. 1,4,3,6-dianhydrosorbitol monostearate. IFE somewhat thicker than normal; cell margins are faintly discernible because of slight intercellular edema.

FIGURE 4.—Mouse #9002. Polyoxyethylene-1,4-sorbitan distearate. A distinct, regular IFE hyperplasia; cells and nuclei markedly above normal in size; parakeratosis; pronounced desquamation in stratum corneum.

FIGURE 5.—Mouse #8969. 1,4,3,6-dianhydrosorbitol distearate. IFE shows moderate hyperplasia, which nevertheless is irregular.

FIGURE 6.—Mouse #8995. Poloxyethylene-1,4-sorbitan tristearate. High-degree IFE hyperplasia; cells and nuclei considerably larger than normal; intercellular spaces wide; intercellular bridges well differentiated; no cellular or nuclear atypia.

FIGURE 7.—Mouse #8981. Polyoxyethylene-1,4,3,6-dianhydrosorbitol monostearate. IFE hyperplasia similar to that in figure 6.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Effect of Air Pollutants on Ciliated Mucus-Secreting Epithelium [1, 2]

HERTA M. TREMER, *Ph.D.*, HANS L. FALK, *Ph.D.*, *and* PAUL KOTIN, *M.D.*,[3] *Department of Pathology, University of Southern California School of Medicine, and the Los Angeles County General Hospital, Los Angeles, California*

SUMMARY

The physiologic activity of the respiratory epithelium is adversely affected by chemical agents in polluted urban atmosphere. This is manifested by a slowing of the flow of the mucous stream, alteration in the physical properties of the mucus, and changes in ciliary activity. The response may be quantitated by measuring the flow rate of particles of isolated strips of ciliated mucus-secreting epithelium. While quantitative differences exist in different species of animals, the qualitative pattern is a relatively consistent one. The impingement of chemical agents produces a more intense and longer lasting effect than does the passive flow of agents over the epithelium. The demonstration *in vitro* of slowing of mucous flow can be paralleled by using studies *in vivo* in which abnormal retention of soot can be demonstrated following exposure of intact animals to chemical agents in polluted urban atmosphere. The changes in mucous flow observed during quantitative studies are associated with histologically demonstrable changes in the epithelium. Interference with mucous flow facilitates the accumulation and retention of carcinogenic particulates, so that a biologic environment for their optimum action is created.—J. Nat. Cancer Inst. 23: 979–997, 1959.

THE CILIATED cells and mucous layer are important in the defense mechanism of the respiratory tract. Under physiologic conditions the cephalad flow of the mucous stream prevents the accumulation of particulate matter on the respiratory epithelium. The movement of this stream is primarily related to two factors: (1) The action of the cilia which by virtue of their whiplike activity provide the necessary propelling force and (2) the amount and consistency of the mucus. Irritants in the respiratory environment have been demonstrated to interfere with ciliary activity and flow of the mucous stream, so that the accumulation of particulate matter on the underlying cells ensues. Wharton (*1*), in 1931, demonstrated that ciliary inhibition occurs from both physiologic and

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mechanical causes. Cralley (*2*), in 1942, studied the effects of a broad spectrum of irritant gases on the rate of ciliary activity and demonstrated an inhibiting effect of these agents on ciliary action and flow of the mucous stream. He concluded that the ratio of ciliary response is consistent, though the intensity may vary when methods *in vitro* and *in vivo* are compared. Hilding (*3*), in a series of studies dating back to 1932, demonstrated that a large variety of exogenous agents—chemical, physical, and microbiologic—were capable of interfering with ciliary activity and modifying the flow of the mucous stream.

A comprehensive review of the physiology of the flow of the mucous stream and ciliary action was recently published by Dalhamn (*4*), who demonstrated that alterations in the density of the mucus and changes in the rate of ciliary action were both significant factors in modifying the physiologic activity of the respiratory epithelium. Krueger and Smith (*5*) have reported studies in which they have shown that positively charged ions in the air decreased ciliary activity and slowed the flow of the mucous stream in mammalian respiratory epithelium both *in vitro* and *in vivo*.

Carcinogenic agents have been demonstrated in several areas of our respiratory environment. Waller (*6*), Shabad (*7*), and two of the authors (*8*) have identified benzo[*a*]pyrene in polluted urban atmospheres. The observed increase in pulmonary cancer throughout the world is compatible with a possible role for this and other carcinogenic aromatic polycyclic hydrocarbons, acting in association with respiratory irritants in the pathogenesis of this disease. We have demonstrated that carcinogenic hydrocarbons, particularly benzo[*a*]pyrene, survive in the polluted atmosphere sufficiently long to be breathed by exposed populations (*9*). Because of the theoretical necessity that carcinogenic agents in an adsorbed state be in contact with target cells for a prolonged period, interference with the normal flow of the mucous stream must be postulated as part of the pathogenetic mechanism. Investigations in our laboratory have established that a broad spectrum of irritants is capable of modifying the flow of the mucous stream. In certain specific instances, carcinogenic agents in our respiratory environment have been shown to exert this irritant effect over and above their cancer-producing property. Alternately, simultaneous or sequential inhalation of noncarcinogenic irritants and carcinogenic agents may provide the necessary environment for tumor initiation.

In order to evaluate the role and significance of irritants in our respiratory environment in the pathogenesis of lung cancer, we instituted a series of studies ultimately aimed at quantitating the effect of inhibition of ciliary activity and flow of the mucous stream on retention and distribution of particulate matter on the respiratory epithelium. The present report is concerned with the development and standardization of methods for the quantitative determination of rates of flow of mucus following exposure of ciliated mucus-producing epithelium to irritants in the polluted atmosphere. In addition, we have attempted to correlate our physiologic

findings with morphologic changes in the respiratory epithelium. In an ensuing paper, the effect of cigarette smoke (another source of irritants in our respiratory environment) will be described, and compared with the response to irritants in polluted air.

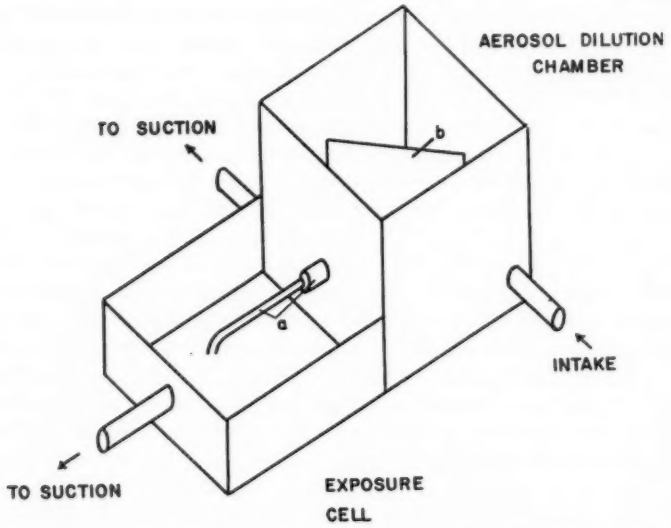
METHODS

The measurement of mucous flow was first undertaken with the use of exteriorized and tracheobronchial segments *in situ* in anesthetized rats or rabbits. The observations reported here are exclusively limited to changes in the rate of mucous flow. Changes in mucous secretion will be discussed in a future publication. Parallel studies were also performed using isolated tracheobronchial segments from rats and rabbits under otherwise identical laboratory circumstances. It was soon found that the data were comparable both qualitatively and quantitatively. The maintenance of constant temperature and humidity in the presence of artificially introduced environmental agents was a difficulty that we sought to overcome by the substitution of a mucus-secreting ciliated epithelium from a cold-blooded animal. We selected the ciliated epithelium of the esophageal tract of the frog to replace the mammalian tracheobronchial segments in our experiments. Parallel studies using isolated mammalian epithelium and amphibian epithelial surfaces established that the latter did not adversely affect either our qualitative findings or the extrapolatability of data. The similarity of effects is graphically presented in the ensuing publication in which the response of the two types of epithelium are shown after exposure to cigarette smoke (*10*). Additional advantages accrue from the use of frog epithelium and include: (1) Prolonged survival of the epithelial segments so that cumulative and relatively long-term studies are possible; (2) relative ease in technical manipulation; and (3) consistent reproducibility of data.

Leopard frogs (*Rana pipiens*) were pithed and the esophagus and adjoining portions of the ciliated intestinal tract were excised. The tissue specimens were opened longitudinally, stretched to natural length, and fastened to a cork board with short pins. The isolated epithelial segments were exposed to aerosols, of the agents under study, in a specially designed Lucite exposure chamber. The details of construction are shown in text-figure 1. Exposure of the epithelium was accomplished in one of two ways—by passive flow over the epithelium (by-pass) or by direct impingement. As shown in text-figure 1, slight modification of the chamber permitted it to be utilized for either method.

So as to facilitate the comparison of the effect of the various pollutants and irritants studied in the laboratory, the concentrations of the agents are reported in terms of total weight of the aerosolized material to which the epithelium was exposed. Exposure of the epithelium was for a period of 2 seconds unless otherwise noted. We are aware that the response

of the tissue does not reflect exposure to the total dose applied; rather only that portion in immediate contact produces the effect. The least response occurs during by-pass exposure, followed by a greater response on exposure by impingement, whereas a true quantitative response occurs when agents are applied directly to the tissue by the microdrop technique described. Aerosols were introduced into the Lucite chamber and permitted to pass over the epithelial surface in an upstream direction at a height that prevented direct impingement. The aerosols were introduced at a constant velocity of air flow (27 ml./sec.) for predetermined time intervals. This by-pass or passive passage method represented an attempt to duplicate the circumstances *in vivo* of epithelial exposure that exist in



TEXT-FIGURE 1.—Chamber for exposure of epithelium (to scale)—(*a*) removable glass section used for impingement of aerosols; (*b*) diagonally placed Lucite baffle section which facilitates mixing and dilution of test agents with air.

the greater part of the tracheobronchial tree. The second method consisted of impinging the aerosols directly on selected areas of the epithelium by means of an angled section of glass tubing, after which secondary passive involvement of the surrounding areas occurred. This technique reproduced the situation "*in vivo*" as occurring at bifurcations and angulations of the respiratory tract.

After exposure by impingement, it should be emphasized that measurement of particle transport is made on the epithelial segments immediately adjacent to the site of impingement. In the area of actual impingement, the effect is influenced by both the mechanical effect of impaction and the drying. Transient immobility frequently resulted at the impingement site. Particle transport does not occur until such time as mucus from

the surrounding area travels over the test area or mucous secretion is restored locally.

The rate of flow of the mucous stream, as represented by the transport of soot particles on the epithelial surface, was measured in a specially constructed Lucite observation chamber, 11 × 7 × 4 inches, with removable lid section, 6 × 4 inches (fig. 1). For mammalian species, the chamber was kept at 37° C. by means of a 20-watt bulb. The relative humidity was kept high by covering the bottom with moist gauze and by bubbling compressed air through a beaker of water by means of a gas dispersion tube. Humidity alone was controlled when frog epithelium was being used.

A solution of fresh egg white and Locke's solution (1:1) maintained at either 37° C. or at room temperature, as indicated, was used to cover the tissue during intervals between tests. The epithelium was rinsed with isothermic, aerated Locke's solution before and after experimental use.

Determination of rate of flow of the mucous stream.—Particles of carbon black were dropped from a small camel's-hair brush on to the mucous stream covering the ciliated epithelium. The transport of the carbon particles was observed at a magnification of 120 through a stereomicroscope fitted with oculars scaled with 10 divisions per millimeter. Travel of the particles over the scaled area was timed by a stop watch, and the rates were routinely expressed in distance traveled per time unit, *i.e.*, millimeters per minute. The rates reported represent the average of 10 individual readings.

Control studies.—Technical considerations precluded the simultaneous use of any portion of the tissue within the chamber for control determinations. Division of the tissue into two longitudinal strips, with one as the test and the other as the control epithelium, also proved to be infeasible. After establishing that the uniformity of the flow of the mucous stream at a constant level was maintainable for hours, control readings were taken prior to the first application of the test material. The epithelium was considered ready for exposure to the test agents only after constant and uniform rate of particle transport could be maintained for 40 to 50 minutes. The uniformity and consistency of mucous flow during the control period prior to exposure of the epithelium will be discussed in the section devoted to the typical response of the epithelium after exposure. During the entire experimental procedure, the maintenance of constant environmental conditions was recognized as being critical. We were diligent in the application of test agents so as to be assured that they constituted the only variable introduced. Within the limits of technical feasibility, the experimental effects observed could be truly related to the agents being tested. Full recognition exists that the meaningfulness of our data is a function of the validity of our controls. Two or three control readings usually sufficed to define a base-line level of particle transport for each tissue.

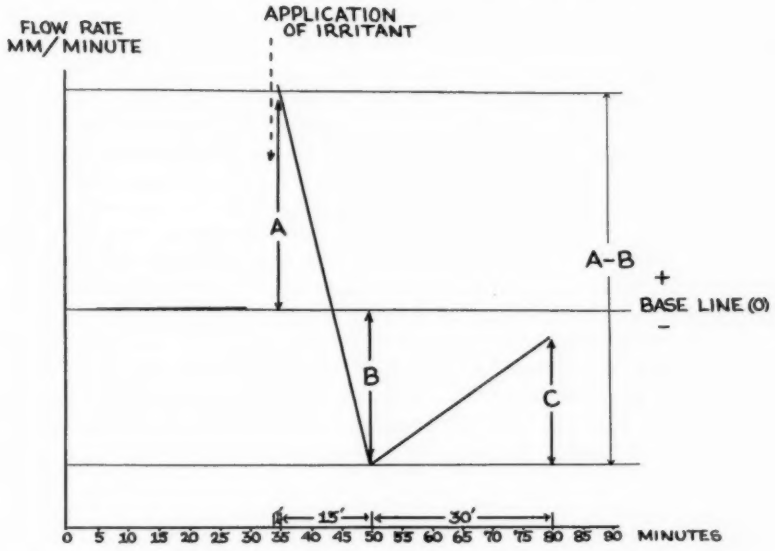
In the instance of natural smog, exposure took place immediately outside the laboratory window. In all instances, the concentration of

the oxidant in the immediate area was at the symptom-producing level (eye irritation) and measured between 0.2 and 0.5 ppm total oxidant as measured by the KI method (*11*).

Measurement of particle transport.—The fastest, uniformly moving particles axially located were selected for determining the rate of flow of the mucous stream and ciliary activity. Peripherally located particles were rejected because of the artifact introduced by the edge of the tissue. After exposure in the Lucite chamber, shown in figure 1, particle transport was studied in the observation chamber shown in text-figure 1. The response of the ciliated epithelium was studied over a period of ¾ of an hour. The rate of mucous flow was determined at 2- to 3-minute intervals during the first 10 minutes subsequent to exposure to smoke. After the first 10-minute period, flow rates were measured at progressively increasing intervals. Three phases characterized the pattern of response of the epithelium. The first was one of a sharp increase with the peak being reached in a few seconds. This initial response was measured 1 minute after exposure and will be referred to as *A*. This was followed by the second phase characterized by a sharp decrease in the flow rate with the base-line or control-level flow rate being crossed approximately 3 minutes after exposure of the epithelium. The decrease in flow rate progressed, although the rapidity of decrease tapered off 10 minutes after exposure. The lowest point of decrease in rate of particle transport was noted 16 minutes after exposure of the epithelium. This 16-minute response will be referred to as *B*. A recovery period of approximately 30 minutes' duration ensued, at the end of which the rate of particle transport either returned to the original base line or a new uniform level of particle flow rate resulted. The measurement of the response at 46 minutes after exposure is referred to as *C*. This is graphically shown in text-figure 2. In successive exposures *A* was related to the previous level at *C*.

Soot retention in vivo.—To complement the experiments *in vitro*, we studied the effect of inhalation of smog on the retention of carcinogenic soots in the respiratory tract of rabbits. An experimental protocol was used that permitted the quantitation of retained soot after predetermined periods of exposure to smog and varying periods of recovery prior to soot inhalation. An inhalation bubble was designed that permitted the simultaneous exposure of 6 rabbits to soot (fig. 2). We anticipated that any abnormal retention of soot would reflect interference with the normal defenses of the respiratory epithelium. The techniques used in the gravimetric determination of the retained soot have been detailed in our report on human lung studies (*12*).

Histologic studies.—At appropriate intervals, sections of ciliated mucous epithelium were taken and prepared for histologic examination. The degree of activity of goblet cells and the intensity of mucous secretion were noted. In addition, other changes in either the respiratory lining cells or subepithelial tissues were sought. Both routine hematoxylin and eosin and special stains were used.



TEXT-FIGURE 2.—Characteristic response of mucous flow to test agents measured at 1, 16, and 46 minutes after exposure. A = effect after 1 minute; B = effect after 16 minutes; C = effect after 46 minutes; $A - B$ = decrease of flow between peak of stimulation and maximum inhibition. *Note* uniform base-line flow rate between 5- and 30-minute intervals during which repeated readings were made. Text-figure 1 A and B in the ensuing paper present the specific data from which this text-figure is derived (*10*).

RESULTS

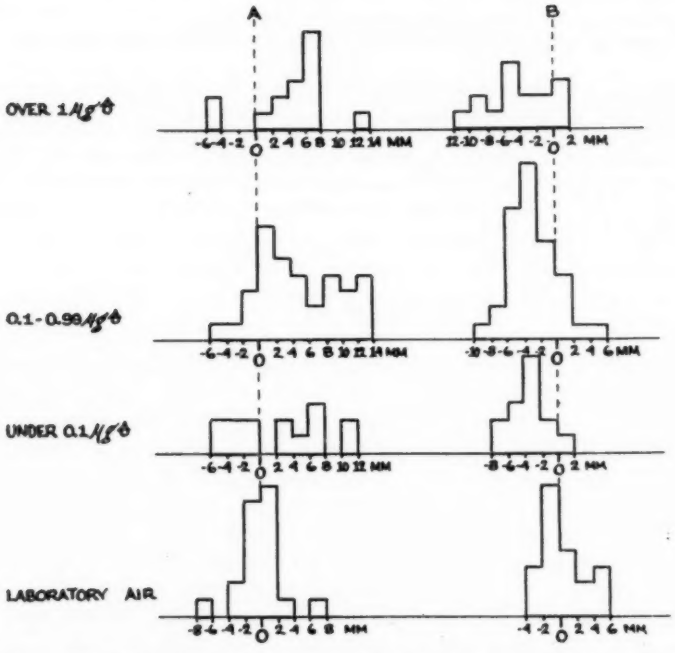
Quantitative studies were first performed with aerosols produced by reacting gasoline with ozone. So as to facilitate chemical studies, hexene-2 was used instead of gasoline as the hydrocarbon source early in our studies. Smogs referred to throughout the text were formed by reacting ozone with hexene-2. This reaction must be meticulously controlled in that the hazard of explosion is great. Ozone was produced by utilizing the techniques of Henne (*13*) and Smith (*14*). The oxidant type of smog resulting has been under intensive study in our laboratory for several years, and its properties have been reported in detail (*15*). The chemistry of the reaction and the identification of its products were first described by Shepherd and his coworkers (*16*), Haagen-Smit (*17*), and Weaver (*18*). In addition to studies with the smog that was produced, ozone and other individual components, and allied compounds have also been investigated. The latter were reagent-grade chemicals. Finally, naturally occurring smog was studied when atmospheric conditions permitted. The findings resulting from the use of synthetic smog, shelf reagents, and natural smog will be discussed individually.

Exposure of the epithelium was by both the by-pass and impingement technique whenever possible, although this was not so in every instance. In all experiments, the various phases of response are related temporally to the time of application of the test material. As previously noted, concentrations will be reported in the quantity or dose of aerosol used so as to facilitate comparison of the effect of the various irritants used.

Ozonized Hexene-2

After the determination of the control or base-line level of mucous flow, the epithelium was exposed to an aerosol of ozonized hexene-2 for 2 seconds, following which it was immediately removed to the observation chamber for measurement of the rate of particle movement.

A comparison of the effect of smog and laboratory air on particle transport when the epithelium was exposed by the by-pass technique is shown in text-figure 3. Various levels of smog concentration were studied and they are arbitrarily divided into quantities of more than 1 μg., 0.1 to



TEXT-FIGURE 3.—Comparison of the effect of smog on rate of mucous flow applied as by-pass in varying concentrations; δ = oxidant. The abscissa records the change in rate of mucous flow compared to the control reading rate "0" expressed in mm. per minute. Increase in the rate of flow is recorded to the *right* of the "0" reading while decrease is noted to the *left*. The ordinate represents the number of individual determinations with each 5-mm. square representing a single determination. Repetitive, identical responses are superimposed one above the other.

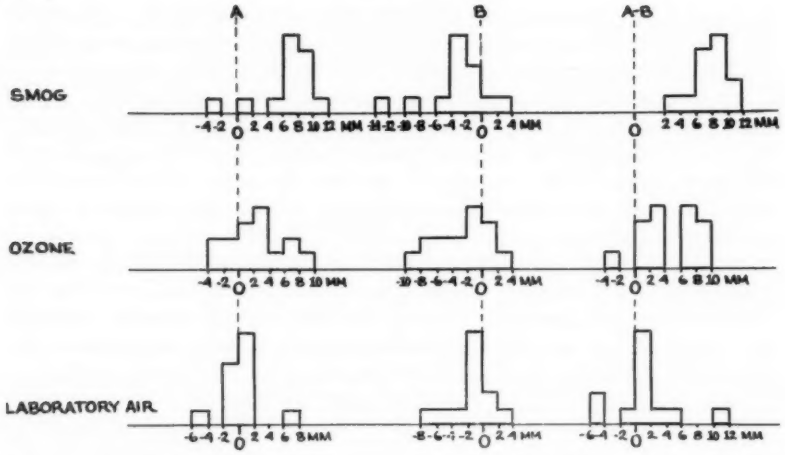
1 μg., and less than 0.1 μg. of oxidant expressed as H_2O_2. It will be noted that at all three concentrations "A" is characterized by an accelerating effect on the particle transport with the effect being minimal at the lowest concentration and greatest at the highest concentration. "B" shows inhibition which is essentially constant at all three concentrations of the pollutant applied. We see thus that while greater concentrations of pollutants appear to be necessary to produce the initial effect of acceleration of the rate of mucous flow, inhibition occurs with relatively equal intensity independent of the concentration of the pollutant. A control study using laboratory air shows virtually no effect, either in initiating stimulation or producing an inhibition of particle transport.

Morphologic changes characterized by alterations in mucous secretion were observed and correlated with the physiologic determinations. The role of changes in the consistency of mucus though recognized as a factor in altering the rate of particle transport will not be discussed at this time.

When the epithelium was exposed to smog by the method of direct impingement, the qualitative pattern of response paralleled that seen with by-pass application; however the results at "A" and "B" were of greater intensity. The application of pollutants by impingement tended to enhance or exaggerate the effect of increasing concentration of pollutants introduced into the chamber. The exaggerated response is in all probability due to the more intimate contact of the aerosol with the tissue and its almost immediate diffusion into the adjacent tissue.

Effect of Ozone

As ozone is one of the two constituents used in producing smog, it was studied in three arbitrarily established concentration ranges of under 1 μg., 1 to 4 μg., and over 4 μg. of oxidant. The techniques of application were similar to those of smog. As in the case of smog, ozone impingement resulted in greater stimulation at *A* than did ozone by by-pass. The latter showed only a very slight stimulation in the middle concentration range. When over 20 μg. oxidant were brought in contact with the tissue, either as by-pass or impingement, the rate of mucous flow dropped immediately, *e.g.*, initial stimulation was observed. The inhibition continued and reached its maximum 16 minutes after application. No significant differences were observed between the two modes of application in any of the concentration ranges during this phase. A comparison of the effects of smog, ozone, and laboratory air on the rate of particle transport is shown in text-figure 4. The method of exposure is that of impingement. It will be noted that the effect at *A* on the epithelium is much greater from smog than it is from ozone while only slightly greater at *B*. The small difference observed at *B* in text-figure 4 becomes obliterated when the data from a large number of determinations are analyzed. The effect of laboratory air on the epithelium following impingement is similar to that seen in the by-pass experiments. No statistically significant response is noted.



TEXT-FIGURE 4.—Comparison of smog, ozone, and laboratory air on rate of mucous flow when applied by impingement. Exposure was for 3 intervals of 2 seconds, each with a 2-second rest between exposure periods for a total of 6 seconds of exposure. The abscissa records the change in rate of mucous flow compared to the control reading rate "0" expressed in mm. per minute. Increase in the rate of flow is recorded to the *right of* the "0" reading, while decrease is noted to the *left*. The ordinate represents the number of individual determinations with each 5-mm. square representing a single determination. Repetitive, identical responses are superimposed one above the other.

A comparison of the effect of natural smog, artificial smog, and laboratory air on particle transport is shown in text-figure 5. All three were studied after a 5-second application by by-pass. It can be readily observed that the effect of natural smog is qualitatively similar to that observed with artificial smog. The control findings with laboratory air are as described previously.

In view of the similar qualitative response observed after the exposure of epithelium to artificial smog, natural smog, and ozone, we undertook to investigate the effect of various agents in the reaction products of ozone and hydrocarbons, as well as other chemical compounds of pollution significance incidental to the manufacture, distribution, and use of certain industrial products. The reagents selected for study are listed in table 1 and the median response at *A* and *B* is presented in tabular form.

A significant factor in determining the response of the epithelium to individual chemical compounds, which are typically present in polluted air, was their solubility in water. This was so for acids, peracids, aldehydes, or bases. While absolute uniformity was lacking, these latter groups generally produced inhibition and slowing of mucous flow both at *A* and *B*.

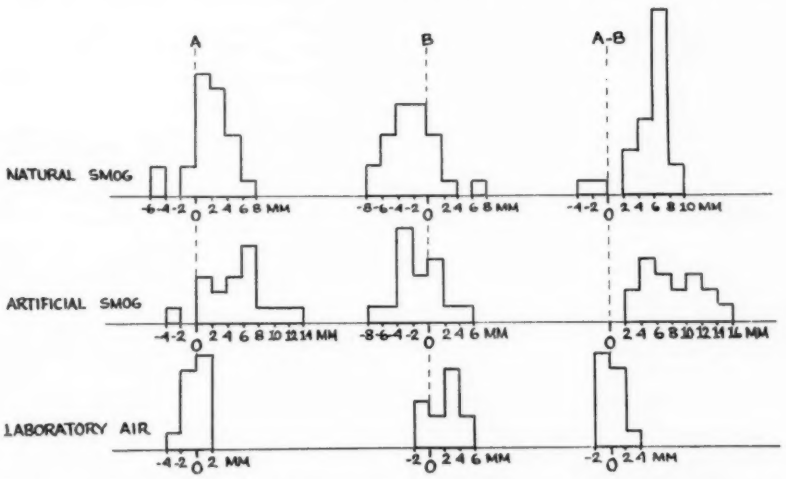
With halogenated hydrocarbons, the response was uniformly minimal despite increasing the degree of halogenation from methyl chloride to carbon tetrachloride. No more than a 10 percent change from the control level could be detected at either *A* or *B*.

The concentrations selected for the testing of these agents were arbitrarily chosen and the possibility exists that subthreshold concentrations may have been used and that the responses might differ qualitatively or quantitatively after exposure to greater concentrations. It was noted that the response to increasing concentrations of individual compounds at *A* and *B* was similar for nearly all groups—specifically a lower rate of mucous flow.

It is not possible at present to correlate chemical structure with the type of response of the ciliated epithelium. Chemical agents which produce changes in *p*H, such as acids, peracids, oxides of nitrogen, and bases, produced definite inhibition at *A* and *B*, particularly at the higher concentrations. Epoxides also produced this type of response with increasing dose, although no change in *p*H occurred.

We conclude from these observations, however, that individual components of polluted air can give contrasting responses, depending on the concentration of the chemical agents. The over-all effect, however, tends to be one of inhibition which increases with increasing concentration.

Rabbits were exposed, in groups of 6, to commercial soot after differing periods of exposure to synthetic smog containing 1.5 ppm total oxidant and were then allowed varying intervals of recovery in a smog-free environment. The simultaneous exposure of the 6 animals to smog and then to soot was undertaken to reduce to a minimum the artifact incidental to individual animal variation. The amount of soot was determined gravi-



TEXT-FIGURE 5.—Effect of natural smog, artificial smog, and laboratory air on rate of mucous flow when applied as by-pass for 5 seconds. The abscissa records the change in rate of mucous flow compared to the control reading rate "0" expressed in mm. per minute. Increase in the rate of flow is recorded to the *right* of the "0" reading, while decrease is noted to the *left*. The ordinate represents the number of individual determinations with each 5-mm. square representing a single determination. Repetitive, identical responses are superimposed one above the other.

TABLE 1.—Median response of epithelium at *A* and *B* after exposure to chemical reagents in mg. quantities, in or related to compounds found in polluted urban air.

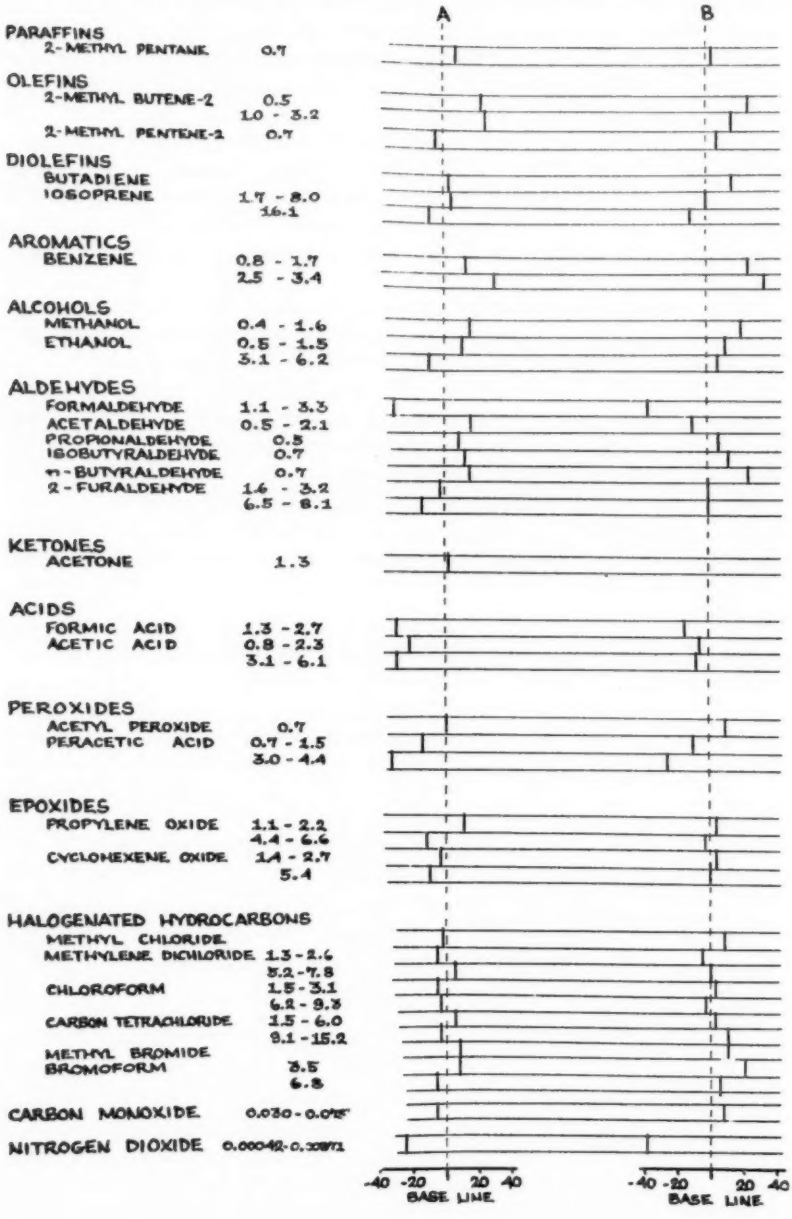


TABLE 2.—Effect of exposure of rabbits to smog (ozonized gasoline 2 ppm) on retention of inhaled soot

	Smog exposure (*hours*)	Room air (recovery) (*hours*)	Soot exposure (*hours*)	Room air (recovery) (*hours*)	Soot in lung (*mg.*)
Control	0	0	1	0	3. 7
Animal #1	1	0	1	0	7. 9
Animal #2	1	0	1	24	5. 4

TABLE 3.—Effect of exposure of rabbits to smog, on retention of inhaled soot following varying recovery periods from smog

	Smog exposure (*hours*)	Room air (recovery) (*hours*)	Soot exposure (*hours*)	Room air (recovery) (*hours*)	Soot in lung (*mg.*)
Control	0	0	1	0	1. 1
Animal #1	1	4	1	0	7. 4
Animal #2	1	8	1	0	5. 2
Animal #3	1	24	1	0	2. 0

metrically. The actual data from typical experiments are given in tables 2 and 3.

Morphologic Studies

We have previously reported that the response of ciliated mucus-secreting epithelium to irritants is characterized initially by an increase in the activity and number of goblet cells (*19*). This may persist until desquamation of the superficial epithelium occurs. A change in consistency and character of mucus also accompanies quantitative changes in secretion. This may be qualitatively demonstrated in histologic sections prepared after varying conditions of epithelial exposure. The simultaneous fixation, dehydration of tissue, and staining for mucus with PAS and mucicarmine under rigidly controlled conditions, at least qualitatively, corroborate the observations of changes in mucus made during studies *in vivo* and *in vitro* (figs. 3, 4, and 5).

DISCUSSION

Before discussing the role and action of irritants in polluted air in the pathogenesis of pulmonary cancer, certain conclusions may be derived from the data. Despite certain quantitative differences in the response of the epithelium to irritants in various concentrations, it is immediately apparent that interference with the flow of the mucous stream as determined by particle transport represents essentially a nonspecific effect in relation to the chemistry of the agents studied. While it is impossible at this time to gauge accurately the effect of systemic or neurohumoral factors in the response of the epithelium to irritants, it is obvious that

changes in the flow of the mucous stream are governed to a remarkable degree by the local properties of the tissue. As observed by Dalhamn and confirmed by us, no linear relationship can be established between the rate of the flow of the mucous stream and the percentage of ciliated cells functioning and the intensity of their beat. One additional factor readily observable during the experimental circumstances reported is the effect of both the quantity and consistency of the mucus. The chemistry of the mucus and the alterations in response to the effect of irritants is in need of elucidation.

Although the initial response of the epithelium to many irritants under practically all circumstances of exposure is one of stimulation, the effect is a transitory one of relatively short duration. While one may postulate that this is an attempt on the part of the epithelium to rid itself of adverse environmental substances, even under the most advantageous circumstances this is accomplished poorly, if at all. More significant from a temporal point of view is the consistent inhibition of the flow of the mucous stream that immediately follows the transient phase of stimulation. As will be noted from our data, it not only persists for a greater period of time, but is less sensitive to changes in both the irritants themselves and the amounts applied.

From the observation of the epithelial response, we may conclude at this time that first, the most sensitive indicator to variations in both the nature and quantity of the irritant is the response at *A*. The most meaningful response in terms of adverse biologic effect obviously occurs at *B*.

In addition to the acute or immediate effects resulting from interference with the flow of mucus, it is our belief that this phenomenon is significant in the pathogenesis of pulmonary cancer. The hazard incidental to exposure to polluted air, such as occurs in Los Angeles and other urban communities, is a multiple one. As already noted, the carcinogenic agents have been identified and quantitated in urban air. Second, their abnormal retention is facilitated by irritants primarily of a noncarcinogenic nature. Third, increased time is made available for the action of agents in liberating adsorbed carcinogens from the particles to which they are adsorbed. Fourth, periodic desquamation of the superficial epithelium permits direct apposition of the carcinogenic agents to the basal layer of cells in the tracheobronchial epithelium.

The links will be described in detail in our discussion of the effect of cigarette smoke on ciliated mucus-secreting epithelium.

CONCLUSIONS

Chemical agents that are present as irritants in the polluted urban atmosphere are capable of interfering with the physiologic activity and normal defenses of the respiratory tract. While the studies described here were primarily performed on the ciliated tissue of the esophagus of

the frog, the applicability of our findings to the epithelial lining of the tracheobronchial tree of mammals was routinely checked and confirmed by repeated parallel studies. This is accomplished by modifying the rate of flow of the mucous stream overlying the epithelial cells. After exposure of the epithelium, the initial response is usually one of a transient increase in mucous flow. This is rapidly superseded by a slowing in the flow rate of mucus, which is followed by an increase in the flow rate toward the base-line rate of flow. This effect is fundamentally a nonspecific one in that an extremely broad spectrum of essentially unrelated chemical compounds can produce the changes. Quantitation of the effect can be accomplished by measuring the rate of transport of soot particles deposited on isolated strips of ciliated mucus-secreting epithelium in exposure chambers, under controlled conditions. The response is not a species-specific one in that it occurs on the respiratory lining of all mammals studied and the esophageal lining of the frog (*Rana pipiens*). The most sensitive phase of the response is that of initial transient increase in flow rate, which tends to vary in intensity with the exposure agent. Slowing and recovery are relatively constant, more persistent, and, to a large degree, independent of the agent being tested. Direct impingement of the agents on the epithelial surface produces a more quantitatively intense, though essentially qualitatively similar, response.

In addition to changes in the rate of mucous flow, changes in the consistency of the mucus occur after exposure of epithelium to irritants. These changes have not as yet been studied, although they are important in determining the flow rate of mucus.

Morphologic studies carried on simultaneously with the physiologic studies revealed a marked increase in the number and activity of the goblet cells. The intensity of histopathologic changes parallels the intensity of the abnormal physiologic response.

Interference with mucous flow facilitates the accumulation and retention of particulate matter, which is deposited on the epithelium of the respiratory tract of intact animals. From the viewpoint of both the short-term acute effect and the long-term carcinogenic effect, our data appear to have significant adverse biologic implications.

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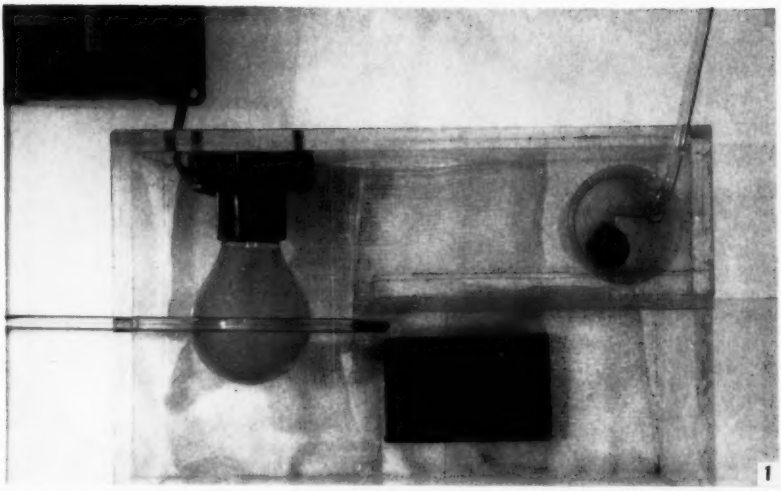
PLATE 92

FIGURE 1.—Lucite chamber used for measuring rate of mucous flow.

FIGURE 2.—Plexiglass bubble used for inhalation studies on rabbits.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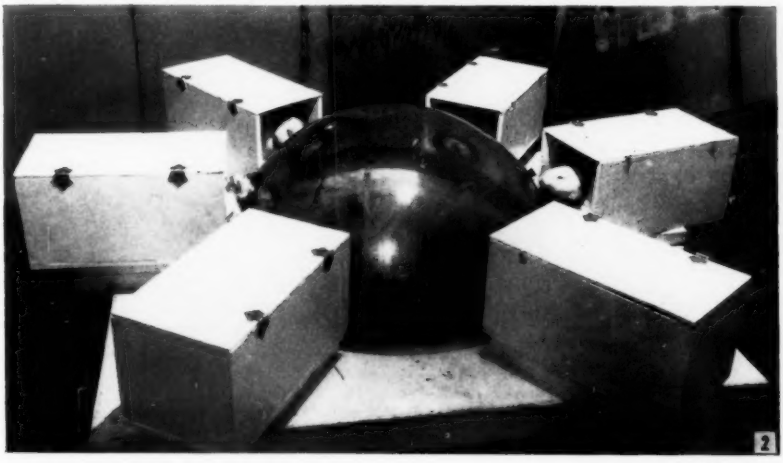
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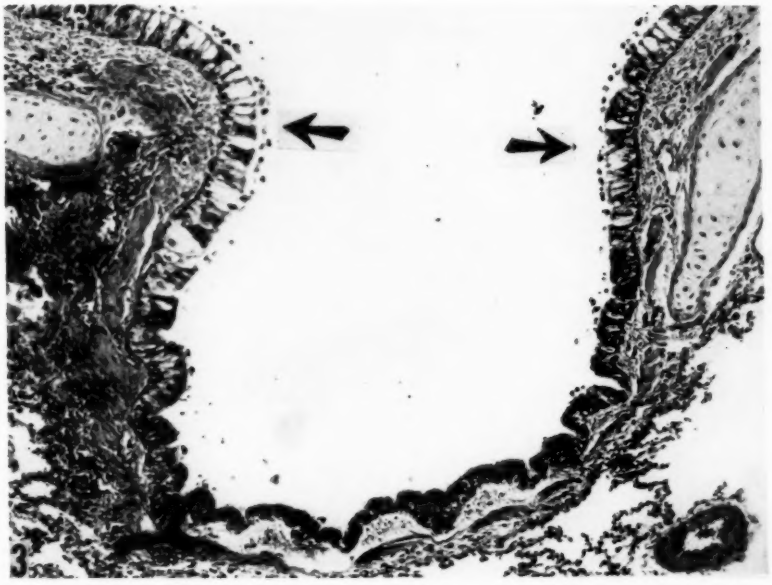
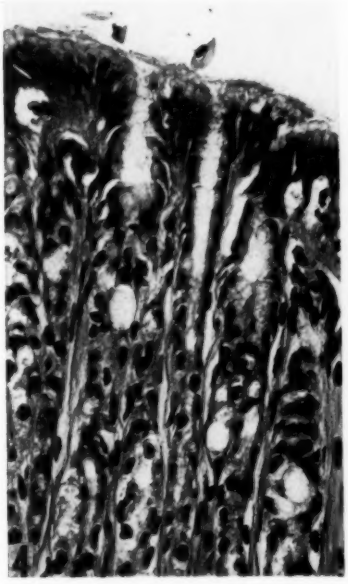
PLATE 93

FIGURE 3.—Section showing increase in goblet-cell activity at impingement site in bronchial segment from rabbit after 1-hour exposure *in vivo* to smog. *Arrow* points to sites opposite cartilage where effect is evident. Nonimpinged area at base of lumen essentially free from effect. × 250

FIGURE 4.—Section from isolated esophageal segments from frog. Control showing essentially normal epithelium. × 250

FIGURE 5.—Section from isolated esophageal segments from frog. Epithelium after 30-second exposure to smog, 1 hour after exposure. × 250





Effect of Cigarette Smoke and Its Constituents on Ciliated Mucus-Secreting Epithelium [1, 2]

HANS L. FALK, *Ph.D.*, HERTA M. TREMER, *Ph.D.*, *and* PAUL KOTIN, *M.D.*,[3] *Department of Pathology, University of Southern California School of Medicine, and the Los Angeles County General Hospital, Los Angeles, California*

SUMMARY

One of the properties of cigarette smoke is its ability to interfere with the normal function of ciliated, mucus-secreting epithelium. The effect is manifested primarily by a slowing of the rate of mucous flow, quantitative changes in mucous secretion, and qualitative changes in mucous consistency. The effect of cigarette smoke is dependent upon its aerosol nature as well as its chemical composition. Reduction in the quantity of aerosols in cigarette smoke results in a reduction in the intensity of the response of the epithelium. At all levels of aerosol production, however, a response is elicited. Filtration of smoke is capable of obviating the response only when it is sufficiently efficient to retain all particulate matter so that gas-phase material alone results. This gas phase, in the concentrations studied, produces no significant effect on the epithelium. It is well to emphasize that during this state the term "smoke" is not applicable. A study of the individual components in cigarette smoke demonstrates that both alkaloids and other chemical compounds are effective in producing the response. The application of an aqueous solution of tars, which were collected from filters, results in the inhibition of mucous flow typically seen with whole cigarette smoke. The accumulation and retention of particulate matter deposited on the epithelium is facilitated by the demonstrated adverse effect of cigarette smoke on the epithelium. This observation suggests a role for cigarette smoking in the pathogenesis of pulmonary cancer.—J. Nat. Cancer Inst. 23: 999–1012, 1959.

IN A previous report (*1*), we discussed the function of the epithelial lining of the tracheobronchial tree in preventing accumulation of inhaled particulates. We presented data which showed that one of the properties of irritants is the ability to interfere with this defense mechanism. Techniques were described whereby the action of irritants may be quantitated by measuring the rate of transport of particles deposited on a mucus-secreting, ciliated, epithelial surface. Data were presented which dem-

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[3] The photographic work was done by Mr. Lloyd Matlovsky, Department of Photography, University of Southern California School of Medicine.

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onstrated that a broad spectrum of chemical compounds in polluted air modified mucous flow on the epithelium (*1*, *2*). We are currently reporting the quantitative effect of (*a*) whole cigarette smoke, (*b*) smoke from filtered cigarettes, (*c*) individual compounds in cigarette smoke, and (*d*) allied compounds on the physiologic activity of the ciliated, mucus-secreting epithelium. The significance of our findings in relation to the pathogenesis of pulmonary cancer will be discussed.

Proetz (*3*), in 1939, demonstrated that cigarette smoke affected secretion and flow of mucus in the respiratory tract. More recently, Hilding (*4*, *5*) has reported studies on the deposition and accumulation of cigarette tar on respiratory epithelium with special emphasis on its possible relation to the development of bronchial cancer. Chang (*6*), in studying the microscopic characteristics of bronchial epithelium of smokers and non-smokers, concluded that the bronchial epithelium from patients with a history of cigarette smoking showed an increase in the number of goblet cells, a large percentage of which were markedly distended. Leuchtenberger (*7*) and her associates, in an experimental study of the cytologic and cytochemical changes in the tracheobronchial tree of mice exposed to cigarette smoke, observed changes in the bronchial epithelium and the peribronchial tissues characteristic of severe bronchitis. Marked proliferative changes in the epithelium accompanied the inflammatory process. They further correlated the cytologic changes with histochemical abnormalities.

In addition to the physiologic studies reviewed, the presence of carcinogenic aromatic polycyclic hydrocarbons has been detected in cigarette-smoke condensates by Cooper and Lindsey (*8*), Latarjet and his coworkers (*9*), Ahlmann (*10*), Lam (*11*), Van Duuren (*12*), and ourselves (*13*). Mellors (*14*) utilized the fluorescent properties of cigarette smoke to study its localization in the respiratory tract of experimental animals. After exposure of the animals to cigarette smoke, he observed an increase in the number of mucus-secreting cells and the accumulation of fluorescent materials first in the mucus and, finally, into the apical cytoplasm of the lining cells.

METHODS

The equipment and techniques used for the exposure of epithelial segments and the measurement of particle transport have already been described (*2*).

By-pass and impingement.—For the exposure of epithelium to aerosols of cigarette smoke, cigarettes were smoked into an exposure chamber, described in a previous article. Fifty ml. of cigarette smoke was collected in the chamber. The smoke was drawn directly into the mixing chamber with displacement of 50 ml. of pre-existing air within the chamber. The total volume of the chamber is 150 ml., so that a ratio of 1:2 of cigarette smoke to air resulted prior to exposure of the epithelium by either impingement or by-pass.

Aqueous solution application.—Cigarettes were smoked in 8 puffs and the water-soluble components were collected in 2 ml. of water. The resulting solution of cigarette smoke was applied in microdrop quantities (5–10 μl.) at the downstream end of the tissue for periods of 2 or 30 seconds. The cigarette-smoke solution was then rinsed off with Locke's solution that was applied to the tissue in a downstream direction. The epithelial segments were then transferred to an observation chamber, previously described. In all experiments, readings of distances traveled were taken at 1-, 16-, and 46-minute intervals after application, as at these time periods the maxima of responses were noted. An initial period of excitation or acceleration of flow of the mucous stream was manifested by an increased rate in transport of the particles along the epithelium. This initial effect at *A* was measured, as noted earlier, 1 minute after exposure. This was followed by an inhibition of the flow of the mucous stream, reflected by a deceleration of particle transport which was determined at the 16-minute interval at *B*. A final period of recovery, *C*, in which there was an increased particle-passage rate in the direction of the base line or control level was measured 46 minutes after exposure.

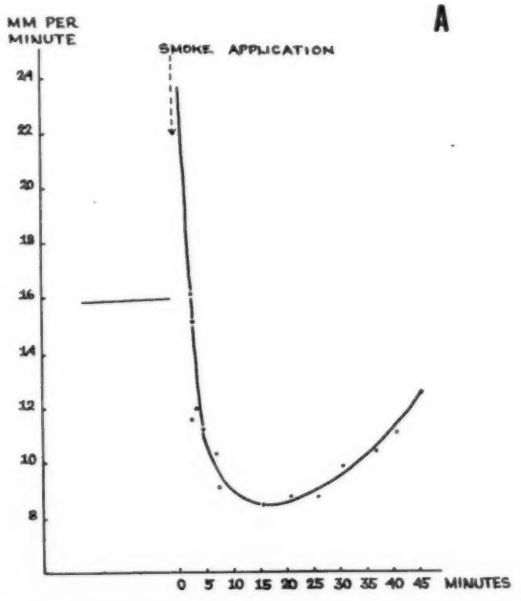
RESULTS

A comparison of the effect of cigarette smoke on the ciliated epithelium of the frog and the trachea of the rat and rabbit was accomplished by exposure of all three epithelial surfaces under identical circumstances (text-fig. 1*A* and *B*). The response was qualitatively comparable at all three phases. The control levels in mammals were one third to one half that observed in frogs, and the magnitude of the response for mammals was also smaller than that seen with frog epithelium. Except for these anticipated quantitative differences, the qualitative response and temporal aspects, as noted, were consistently similar for all three epithelial segments.

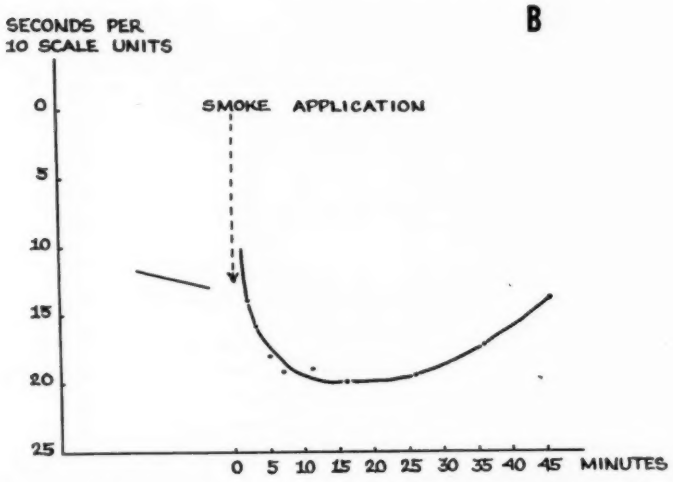
Variations in Relation to Method of Exposure

Differences in the response of the epithelium to smoke in relation to the method of exposure were studied. Aerosols of smoke were utilized by the by-pass method, which has been described, or by the technique of direct impingement. In the latter method smoke is drawn into the mixing chamber, as described for the by-pass exposure; however the diluted cool smoke is pulled into the exposure chamber through a glass tube with a terminal bend so that the smoke stream impinges directly on a small area of the ciliated epithelium (text-fig. 2). Application was usually for 2-second periods. The qualitative pattern of response was identical for both, though the magnitude varied in relation to the application. In addition to these two methods of exposure, we utilized the knowledge that many of the constituents of smoke, responsible for the

epithelial effect, were water-soluble, so that a third method of exposure of the epithelium was introduced: that of direct application of an aqueous solution of smoke. This third mode of application produced a response qualitatively similar to that obtained with the two methods described earlier. The 2-second impingement elicited the strongest degree of stimulation at *A*. Application of an aqueous solution of cigarette smoke for 30 seconds produced the next most intense response, while exposure of the epithelium for 2 seconds by the by-pass technique produced the least effect. Inhibition at *B*, however, was most intense after the application of the aqueous smoke solution. Exposure to cigarette smoke by impingement and by-pass produced inhibition of equal intensity, but of a lesser degree than that seen with the aqueous solution. Recovery at *C* after the 2-second exposure by impingement and by-pass resulted in a re-establishment of the control level of the rate of particle flow. Rarely did the rate of recovery fail to return to the base line. In marked contrast, however, the level during recovery after application of the



TEXT-FIGURE 1*A*.—Response of ciliated esophageal epithelium of frog after application of smoke solution for 30 seconds, as determined by particle transport on mucous stream. *Note* peaks of response at 1-, 16-, and 46-minute intervals. The *solid* line to left of the curve represents pre-exposure behavior determined for a period of 1 hour or longer. The maximum response may be visualized by connecting the end of the control reading and the peak of response 1 minute after application of smoke. The connecting line has been omitted from the graph because the short interval between exposure and response precluded accurate interim readings.



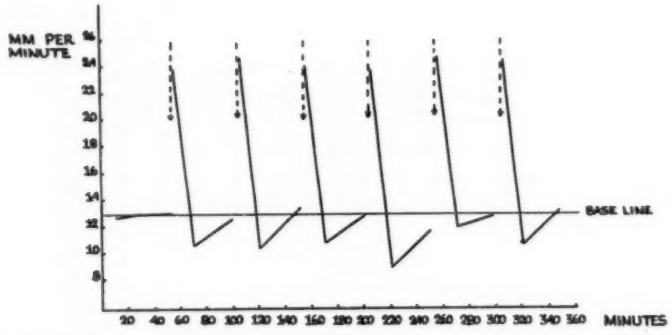
TEXT FIGURE 1*B*.—Response of ciliated tracheobronchial epithelium of rat after application of smoke solution for 30 seconds as determined by particle transport on mucous stream. *Note* peak of response at 1-, 16-, and 46-minute intervals. Criteria for *A* apply here also.

aqueous solution never reached the control base-line flow rate but leveled off at a lower flow rate.

Variations in relation to amount of tar or cigarette-smoke condensate.—In an attempt to determine whether the effect on the epithelium was related to the amount of cigarette-smoke condensate produced, aqueous solutions of smoke from two contrasting cigarettes were studied, one rich in tars, the other poor. The latter yielded only two thirds as much condensate as the former. The cigarettes were otherwise identical in regard to pack, humectants, type of tobacco, and additives. Smoke from the tar-rich cigarette produced a greater degree of stimulation at "A" than did the smoke from the tar-poor cigarette. Inhibition at "B" and recovery at "C" were, however, similar in both cases. It will be noted that this observation, initially made under the circumstances described, persisted throughout all the subsequent studies. Apparently the intensity of the stimulation varies significantly in relation to many factors, including the chemistry of the irritant, the concentration of the irritant, and the mode of application. Inhibition at *B*, however, is only rarely related to the irritants studied and is similarly very little affected by either the type or duration of smoke application.

Effect of Filtration

We also wished to compare the effect of tar-poor smoke due to low tar production with tar-poor smoke due to the use of filtering devices. Again, the water-soluble fractions of the smoke were studied. Of the various



TEXT-FIGURE 2.—Effect of aerosols of cigarette smoke on particle transport after exposure for 2 seconds by impingement method. *Note* constancy of response for duration of 6-hour interval.

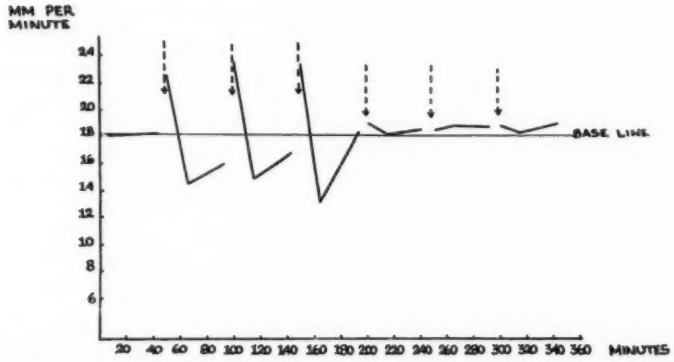
filter types investigated, the first used was designed on the Venturi principle—impingement after acceleration. This eliminated possible differential absorption of individual components. The solution of filtered smoke from both tar-poor and tar-rich cigarettes produced significantly less stimulation at *A* than the unfiltered smoke solution. Inhibition at *B*, however, was constant for both the tar-rich and tar-poor smoke after filtration. This uniform response to the filtered smoke after 16 minutes differed only minutely from the unfiltered control.

To further increase the removal of the solids from cigarette smoke, glass-wool filters were used, in addition to the Venturi filters. The aqueous solution of these doubly filtered cigarette smokes resulted in the typical response, already described. The response, however, occurred only after application of solutions with a higher concentration of condensate from the filtered cigarettes, so that in essence a reduction in the intensity of the response occurs after filtration.

A combination of Millipore filters (#AA, 1-inch diameter) and glass-wool filters was used to obtain a pure gas phase, free of all solids. The gas-phase material was applied for 2 seconds by impingement and for 30 seconds in aqueous solution. In neither instance was any response obtained as neither stimulation nor inhibition could be observed (text-fig. 3).

Effect of Tars Retained in Filters

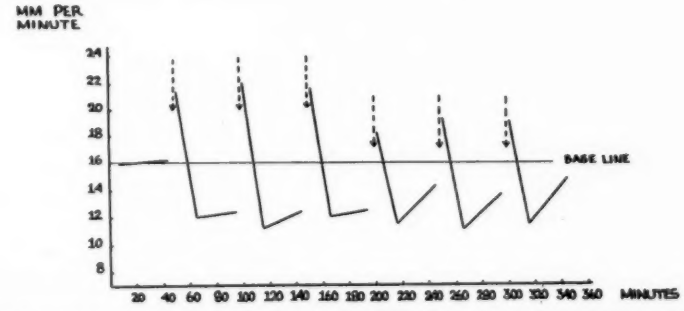
In order to determine the effect of tars retained in the various filter devices, the tars were mechanically removed and shaken with water and applied by the methods described. The magnitude of response at *A* is greater after the use of retained tars from tar-rich cigarettes than from tar-poor cigarettes. In no instance, however, is the intensity of stimulation equal to that observed with whole-smoke solution, as reported earlier. The response at *B* was identical for the tars from both types of cigarettes, nor did it differ from the smoke control.



TEXT-FIGURE 3.—Comparison of response following application of aqueous solution of tars recovered from filter with response following application of gas phase. First three responses following application of tar solution; second three responses following application of gas phase. Samples for both are from tar-rich cigarettes applied to epithelium in aqueous solution for 30 seconds in microdrop quantity.

Effect of Duration of Exposure

We next wished to see what the effect of reducing the exposure time would be on the response of the epithelium. All the applications of smoke solutions described thus far have been of 30 seconds' duration and, as noted earlier, little or no variation could be seen in relation to the type of smoke or mode of application, especially in the 16-minute response of inhibition at *B*. As it was felt that inhibition in all of these instances might be the maximum response capable of elicitation, we applied solutions of smoke from both tar-rich and tar-poor cigarettes for only 2 seconds—$\frac{1}{15}$ of the previous application time (text-fig. 4). Stimulation at *A*



TEXT-FIGURE 4.—Comparison of effect of application of aqueous solution of whole smoke from tar-rich cigarettes applied for 30 and for 2 seconds. First three responses following 30-second exposure and second three responses following 2-second exposure.

after a 2-second application occurred as it did after a 30-second application; however, the magnitude of response was less in the former. The least amount of stimulation occurred after the application of tar-poor smoke for 2 seconds. Inhibition at *B*, however, was of the same magnitude even for the tar-poor-smoke solution when applied for 2 seconds. This in turn was equal to inhibition at *B*, seen after the application of tar-rich-smoke solution for 30 seconds. Recovery at *C* varies with the duration of exposure of the tissue, but never re-establishes the base-line flow rate.

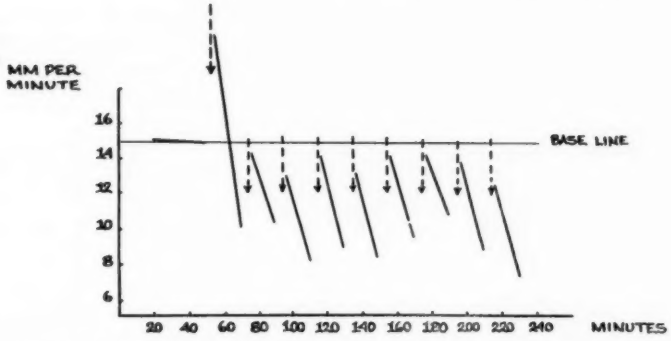
One may properly conclude at this time that major variations in response occur at *A*. Little difference in effect even under the most extreme circumstances could be noticed at *B* (inhibition) or *C* (recovery). The relation of this observation to cigarette-smoke composition in contradistinction to the intensity of exposure independent of smoke chemistry is incapable of delineation at present. The implications of this in relation to the pathogenesis of pulmonary cancer will be discussed.

Effect of Repetitive Exposure of Epithelium

Having established the pattern and intensity of response following single applications of cigarette smoke, we were interested in studying the effect of repetitive exposure on the epithelium in an effort to more nearly parallel the situation existing during actual smoking. In studying the repetitive effect of epithelial exposure, a 20-minute interval was selected as the time between successive applications. This time period precluded the recovery from the preceding application so that the response at "A" from each successive application would immediately follow maximum inhibition. The applications of smoke solution in the repetitive studies were for both 2- and 30-second intervals from both tar-rich and tar-poor cigarettes.

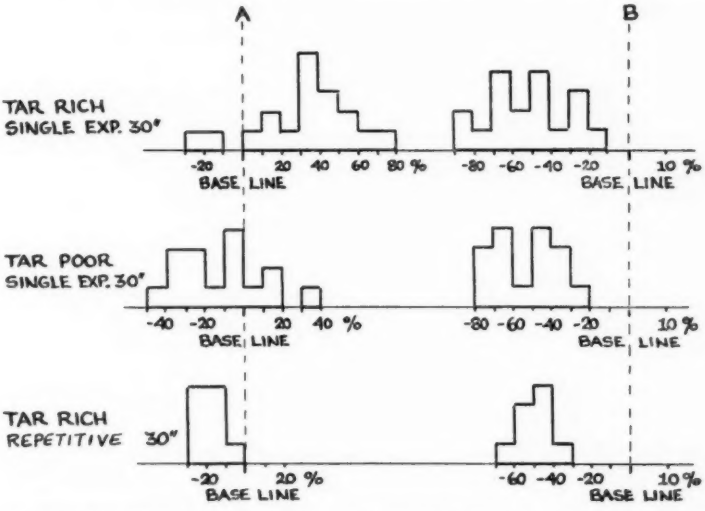
The response at *A* was one of stimulation and acceleration of particle transport in all instances. The most marked effect was observed with tar-poor-cigarette condensate following both 2- and 30-second application. This is in contrast to the effect following single exposure. The response of the epithelium to solutions from tar-rich cigarettes was less than that from tar-poor cigarettes (text-fig. 5). It is significant that none of the 16-minute readings at *B* after repetitive applications showed an inhibition. As in our experiments described previously, *A* and *B* in our repetitive studies were determined in relation to the preapplication level. Each successive application of cigarette smoke to the tissue was made prior to complete recovery of the epithelium to the immediately preceding application. It is well to emphasize at this time that this preapplication level represents the recovery level at "C" in the single-exposure experiments. With repetitive applications, however, the last point of reference before each application is that of greatest inhibition at *B*.

To establish a common point of reference, the preapplication control readings which represent the base-line level of activity were utilized for comparison. The response of the tissue after successive applications



TEXT-FIGURE 5.—Effect of repetitive application of solution of cigarette smoke from tar-rich cigarette for 30 seconds. *Note* initial response at *A* is one of typical stimulation.

of smoke, in the repetitive studies, is expressed in percentage of change from the preapplication base-line readings as shown in text-figure 6. The data obtained from the previously described studies of single exposures to tar-rich and tar-poor cigarettes were similarly recalculated to obtain analogous percentage differences. Stimulation is regarded as having



TEXT-FIGURE 6.—Comparison of responses following single exposure and repetitive exposure of epithelium. *Note* response at *B* is essentially constant throughout. The abscissa records the change in rate of mucous flow compared to the control reading rate "0" expressed in mm. per minute. Increase in the rate of flow is recorded to the *right* of the "0" reading while decrease is noted to the *left*. The ordinate represents the number of individual determinations with each 5-mm. square representing a single determination. Repetitive, identical responses are superimposed one above the other.

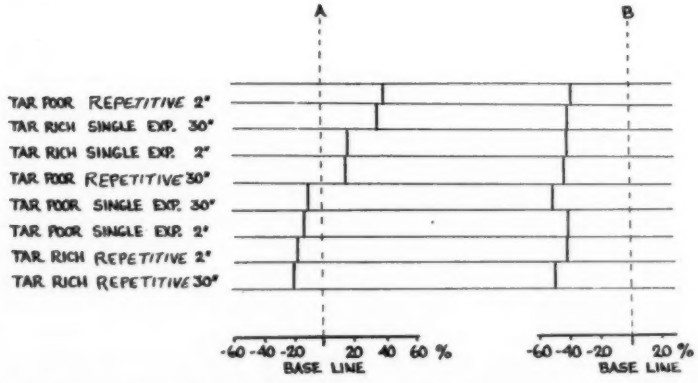
occurred only when the response at *A* results in particle transport at a rate faster than the pre-experimental control level. Thus, acceleration of transport, though invariable at *A* after the application of smoke, reflects stimulation only when pre-experimental control or base-line levels are exceeded.

The median response of the epithelium to both single and repetitive exposures is shown in text-figure 7. Note consistent inhibition at *B* for all circumstances and time intervals of exposure.

An analysis of the response at *A* shows a gradient effect in relation to the type of exposure and smoke preparation used for application. In four instances, the response was a true stimulation. The remaining four combinations, though responding by initial acceleration compared to the control level just prior to application, do so without reaching the base-line level. The response at *B* is consistent, inhibition in all instances.

Effect of Smoke Fractions

Because nicotine is regarded as the most potent agent among those with biologic effects in cigarette smoke, we assayed its effect on epithelium. Cigarette smoke was divided into three fractions by means of solvent partition. The smoke condensate in distilled water was acidified to *p*H 1 and extracted with ether. After evaporation, the ether fraction yielded a lipide-rich, nicotine-poor fraction. The aqueous acidified fraction was made alkaloid with ammonium hydroxide and extracted with ether, giving an alkaloid-rich fraction. The resulting aqueous fraction was produced by driving off the excess ammonia. The maximum response was observed with the nicotine fraction and the least with the water fraction. As always, the differences were noted primarily at *A* with the differences of lesser intensity occurring at *B*. Reagent-grade nicotine yielded a typical pattern of smoke response, both when applied in liquid solution and by impingement as an aerosol.

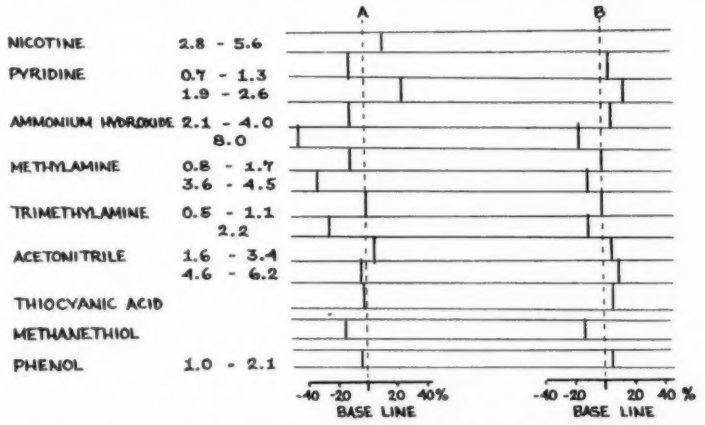


TEXT-FIGURE 7.—Tabulation of median response of epithelium to various smoke preparations for varying durations and frequency of exposure.

Response to shelf reagents.—As with the study of individual compounds significant in air pollution, a series of chemicals either in or related to cigarette smoke were studied. These are listed and their median response is shown in text-figure 8.

DISCUSSION

Before comparing the effects of air pollutants and cigarette smoke on ciliated mucus-secreting epithelium, certain observations in relation to cigarette smoke may be made. Basically, cigarette smoke in all its various fractions, as well as in its entirety, primarily decreases the rate of mucous flow. This predominant, usually persistent, effect occurs after a short preliminary period of acceleration of flow. The deceleration persists for a considerable period and is relatively constant for both whole smoke and its various components. After the maximum slowing, a period of gradual recovery is usually observed. The data *in vitro* demonstrate that the exposure of ciliated epithelium to smoke or its constituents once every 45 minutes results in a slowing of the flow of the overlying mucous stream. Of perhaps greater significance are the data obtained following repetitive exposure of epithelium to smoke. In these circumstances, applications are made at 20-minute intervals at a time when inhibition from previous exposure has reached its maximum. The slowing effect persists. This finding, when considered in terms of the human situation, is compatible with greater retention of particulate matter in the lungs for prolonged time intervals. While in the repetitive studies short transient periods of accelerated particle transport were noted at *A*, the peaks in half the cases were below the base-line control levels established at the start of the experiment. To increase the extrapolatability of the data, we felt it would be desirable to duplicate the exposure of epithelium as it occurs during actual smoking. This could not be accomplished, because repetitive exposures over the short time



TEXT-FIGURE 8.—Median response of epithelium at *A* and *B* after exposure to chemical reagents, in mg., present in or related to constituents of cigarette smoke.

intervals required would be technically difficult to implement and excessively toxic to the excised tissue. Our repetitive studies, we feel, parallel the human situation; although we are, of course, aware of the modifying effects incidental to changes in both duration and intensity of exposure when compared with actual smoking. Further, the use of isolated tissue preparations, rather than the intact animal, is also recognized as a modifying factor.

In view of the adverse effect demonstrated on the respiratory epithelium by cigarette smoke *in toto* and its individual components, the changes in effect as a result of filtration are of great significance. As will be noted from the data, modifications in effect are limited exclusively to acceleration at *A*. This of all phases of the typical response is by far the most transient and least significant from a carcinogenic viewpoint. Filtration, either by static mechanical means or by acceleration and particle deposition, resulted in a smoke that had little or no altering effect on *B* or *C*. Only when the various methods of filtration were applied in tandem so that a gas phase was produced, did we observe a significant change in phases *A*, *B*, and *C*. Under these circumstances, however, the words "cigarette smoke" or "cigarette-smoke condensate" cannot, of course, be used.

Under the experimental conditions of exposure, we have observed that cigarette smoke primarily exerts an inhibiting effect on the flow of the mucous stream of ciliated epithelium. This effect was then quantitated by measuring the transport rate of particles on the stream.

To ascertain the contribution of chemical constituents of the gas phase after efficient filtration, we studied several of these in pure chemical form, by the techniques already described. The pattern of response following the use of each was sufficiently nondescript, so that one can properly assume the gas-phase contribution to the typical response of the epithelium is minimal. This is wholly consistent with our experimental findings. Nicotine was studied in further attempts to understand the effect of smoke and its constituents and to determine the component responsible for the effects. This alkaloid produced a response qualitatively similar to that obtained from the whole smoke. It would appear at this time that nicotine and other alkaloids presumably are at least partially responsible for the whole-smoke effect on ciliated mucus-secreting epithelium.

We noted during our studies on aerosols of air pollutants and aerosols of cigarette smoke that the response to irritants is most variable during *A*. In fact, aerosols of each and their individual components may demonstrate a dose-response relationship only at *A*. At *B* and *C*, the effects are more uniform and appear to be related slightly, if at all, to the dose of the agent, assuming, of course, a threshold has been reached. While the major and most consistent response is that of inhibition of mucous flow as noted at *B*, the initial response of the epithelium to an irritant at *A* is, however, also of significance. The response at *A*, which

is most prominently related to dose, is most readily adaptable for the detection of suspected irritants in the respiratory environment.

A comparison of the effects of smog and cigarette smoke on the epithelium leads one to the obvious conclusion that both agents can produce their effect when present in minute concentrations. Secondly, the response to both at *B* is relatively constant in terms of the intensity, pattern, and time of response. This is manifested by a slowing or deceleration of particle transport along the mucous stream, so that accumulation and retention is maximum after exposure of the epithelium to aerosols of air pollutants and cigarette smoke and their components.

From the viewpoint of the pathogenesis of pulmonary neoplasms, certain previous data are in need of recall. We and others have demonstrated that aromatic polycyclic hydrocarbons, including the carcinogen benzo[*a*]pyrene, as they occur adsorbed on soot in the atmosphere are sufficiently stable to postulate their inhalation by exposed populations. Similarly, carcinogenic aromatic polycyclic hydrocarbons have been identified in cigarette-smoke condensate. Under physiologic conditions, the continuous flow of the mucous stream tends to preclude the accumulation and ultimate prolonged retention of particulate matter on the respiratory epithelium. Normally, retained particulates held within the lungs have been demonstrated by others and ourselves to consist primarily of those ingested by respiratory phagocytes (*15*, *16*). Irritants are present in particulate form both in polluted air and cigarette smoke. Their action in facilitating the localization and retention of particulates serves the carcinogenic process in two theoretical ways. First, it permits abnormally long apposition of the retained particulates to the underlying epithelial cells. Second, it provides an extended time period for the elution of carcinogenic agents from the soot particles to which they are adsorbed, either by inhaled material acting as solvents or by the action of protein solutions as they exist, for example, in the cytoplasm of pulmonary macrophages.

The elicitation of a response to both air pollution and cigarette smoke establishes that from a physiologic viewpoint a hazard exists during exposure to virtually all measurable concentrations of these agents. The apparent nonspecific nature of the response strongly suggests that the physical characteristics as well as the chemical properties of the aerosols are of major significance. In polluted air, both particulate phase and gas-phase materials are effective in interfering with the flow of the mucous stream. In contrast, the disappearance of an adverse effect in studies using the gas phase of cigarette "smoke" emphasizes the critical significance of particles present in whole smoke—this over and above their chemistry. As cigarette smoke and particulates are in essence synonymous, cigarette smoke incapable of producing an effect would be necessarily limited exclusively to its gas phase. Filtration, no matter how effective, can only reduce but apparently never eliminate the effect on the epithelium so long as particulates pass through the filter. The total removal of particulate matter from the products of cigarette com-

bustion would result in a yield which, of course, would no longer be capable of designation as smoke. Our experimental data demonstrate that both air pollution and cigarette smoke, by virtue of their interference with normal function of the respiratory epithelium, qualify for a role in the pathogenesis of pulmonary cancer. Further, the ubiquity of these environmental agents and their etiologic compatability with the epidemiologic data relating to lung cancer place the problem of their significance in the area of contribution to and responsibility for the observed increased incidence. Not to suggest a role for them is irreconcilable with currently available information.

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Effect of Enforced Activity Stress on the Development of Experimental Papillomas in Mice [1, 2]

JOHN A. STERN,[3] GEORGE WINOKUR,[3] DAVID T. GRAHAM,[4] *and* ROBERT LEFTON,[3] *Department of Psychiatry and Neurology, Washington University School of Medicine, St. Louis, Missouri, and Department of Medicine, University of Wisconsin School of Medicine, Madison, Wisconsin*

SUMMARY

The experimental stress of enforced activity was shown to be related to a significant enhancement of papilloma development and death rate. Stress initiated concurrently with application of the carcinogen exerted a more profound effect than was observed in prestressed animals.—J. Nat. Cancer Inst. 23: 1013–1018, 1959.

THE EXPERIMENTAL literature covering the effect of stress on cancer development contains studies which show enhancement (*1–4*), inhibition (*5, 6*), and no effect (*7–10*). The variety of effects may be attributable to the great variety of stresses used, the variety of tumors induced, as well as a function of the differences in experimental organisms used (*11*).

The present study was undertaken to test: (1) the effect of exposing animals to a stressful procedure (enforced activity) on speed of development of methylcholanthrene-induced papillomas, and (2) whether the temporal relationship between initiation of stress and initiation of application of the carcinogenic agent was important.

EXPERIMENTAL PROCEDURE AND APPARATUS

Seventy-six female Swiss white mice between 45 and 50 days of age were used in this experiment. Animals were received at 25 days of age and were randomly divided into 3 groups—1 control and 2 experimental groups. These groups had 25, 26, and 25 mice assigned to them, respectively. Food (Purina laboratory chow) and water were available to all groups *ad libitum* except for the 5-hour period each day during which the experimental animals were subjected to enforced activity.

The 2 experimental groups (Groups II and III) were both subjected to enforced activity 5 hours each day for a total of 75 consecutive days.

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[3] Department of Psychiatry and Neurology, Washington University School of Medicine, St. Louis, Mo.

[4] Department of Medicine, University of Wisconsin School of Medicine, Madison, Wis.

Animals were placed in an activity wheel and forced to run approximately 4,500 feet during each 5-hour period. Group II, the prior stress-carcinogen group, was started in the activity wheel 15 days prior to first painting with the carcinogen. Group III, the concurrent stress-carcinogen group, was started in the activity wheel the day of first application of the carcinogen. Thus, Groups II and III differ only in respect to the time experimental stress was initiated and terminated. Group I, the control group, was not subjected to the experimental stress, except for being deprived of food and water for 5 hours each day, but otherwise was treated exactly like the experimental groups.

In all 3 groups application of the carcinogen was started at exactly the same time—during the resting phase of the hair cycle—when the animals were between 60 and 65 days old. Tumors were induced by application of 3 methylcholanthrene (C 21, H 16, MW 268.34) dissolved in purified benzene to make a 0.6 percent solution. A #2 camel's-hair brush was used to apply the methylcholanthrene to the back of the mouse. In applying the carcinogen, the brush was dipped into and withdrawn from the solution, which allowed any excess to drip off the brush before removing it from the bottle. The animals were painted with methylcholanthrene every other day for a total of 5 applications. The direction of the painting was a fine stroke extending alternately from the nape of the neck to the tail and from the tail to the nape of neck. After each animal was painted, it was placed in a clean cage until dry and was then returned to its home cage.

The prior stress-carcinogen and the concurrent stress-carcinogen groups were stressed from 8:30 a.m. to 1:30 p.m. daily. While these animals were in the activity wheel, the food and water were removed from the control group. All animals were painted with the carcinogen at 4:30 p.m., 3 hours after the animals had been removed from the "stress" situation.

Each group of animals was weighed daily for 7 days before the beginning of the experiment in order to establish a base-line weight. In addition, all 3 groups of animals were weighed twice daily during the 75 consecutive days of enforced activity. They were weighed before being placed in the stress situation and at the end of this stress. After the 75 days of stress, each group was weighed at least once every week.

Tumor evaluation began the day after the last application of the carcinogen. Evaluation was by inspection and palpation, and along a 4-point scale: (0) skin appears to be normal, has no sign of papilloma, skin may be epilated; (1) small papilloma—many times it was possible for the investigator to palpate such papillomas without being able to see them; (2) medium-sized papillomas—the papilloma was visible and 1 to 3 mm. in diameter at the base; (3) large papilloma—over 3 mm. in diameter at the base. Reliability of this scale was established by correlating the observations of 2 independent observers.

The mortality rate in each of the 3 groups was recorded in terms of date of death, and size of tumor at time of death was noted. Since

autopsies were not performed it was not possible to determine the cause of death. Animals that died with grossly observable tumors were retained in the percentage of papillomas developed even after death.

RESULTS

Text-figure 1 depicts the percentage of animals per group that had developed medium or large papillomas as a consequence of the application of the carcinogen. Medium and large tumor percentage was chosen because small papillomas (rating of 1) were often found to regress. As can be seen from text-figure 1, a medium tumor occasionally regressed, but this occurred quite infrequently and mainly during the first 3 months after application of the carcinogen. Using the dichotomous rating of medium and large *versus* small or no papillomas, 2 observers, evaluating independently, obtained 100 percent agreement between their ratings. As depicted in text-figure 1, the concurrent-stress group consistently has a higher percentage of animals with medium and large papillomas than the control group, with the prior-stress group falling between these 2 groups. Statistical comparisons were conducted on every second evaluation. Two by two chi-square tests with medium or large *versus* small or no papillomas as the cutting line were computed for all 3 possible comparisons between the 3 groups. Consistent differences beyond the 0.05 level of significance were obtained between the incidence of papillomas in the concurrent-stress and the control groups. No significant differences in incidence of papilloma development were obtained between the control and prior-stress groups. The last 4 comparisons between the concurrent and prior-stress groups achieve an acceptable level of confidence, with the concurrent-stress group showing consistently more animals with medium or large papillomas than was true of the prior-stress group (table 1).

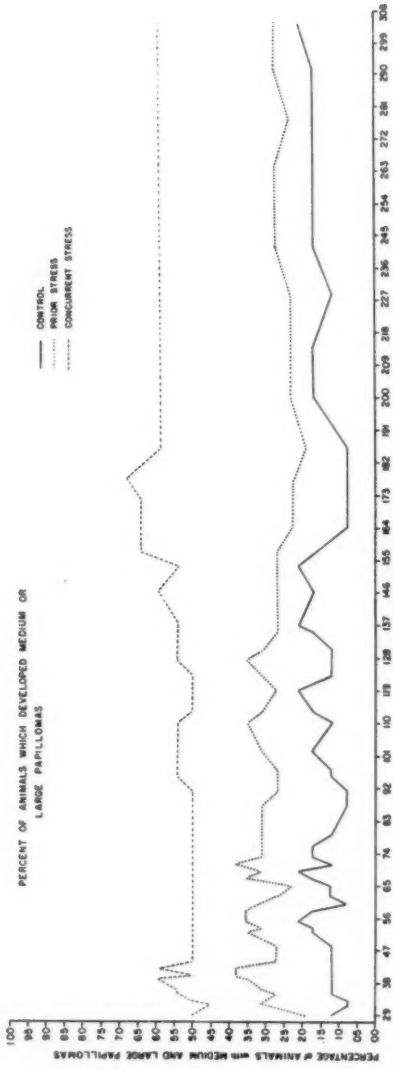
Weight

Animals were weighed twice daily during the period of enforced activity and subsequently once weekly when the papilloma evaluation was made. Except for a transitory weight loss in the prior-stress group during the first week of enforced activity, no weight differences occurred between the 3 groups of animals.

Mortality Rate [5]

A record of the cumulative mortality rate was kept throughout the experiment. At 30-day intervals starting with day 21 after the last day of painting and terminating with day 291, chi-square comparisons were made. Of the 10 comparisons made between the concurrent-stress and the control group, 3 were significant beyond the 0.05 level of confidence. These three significant comparisons fell on 3 successive days of evalua-

[5] These data are available upon request from the authors.


PERCENT OF ANIMALS WHICH DEVELOPED MEDIUM OR LARGE PAPILLOMAS
CONTROL
PRIOR STRESS
CONCURRENT STRESS
DAYS SINCE LAST PAINTING
PERCENTAGE of ANIMALS with MEDIUM AND LARGE PAPILLOMAS

TEXT-FIGURE 1.—Percentage of all animals per group developing papillomas

TABLE 1.—Results of comparisons between groups with respect to incidence of papilloma development

Day since last painting	Groups compared		
	Control-concurrent *P*	Control-prior* *P*	Prior-concurrent *P*
29	.02	—	—
33	.01	—	—
36	.01	—	—
39	.02	—	—
44	.02	—	—
51	.05	—	—
55	—	—	—
60	.01	—	—
64	.02	—	—
69	—	—	—
73	.05	—	—
78	.02	—	—
90	.01	—	—
97	.01	—	—
110	.01	—	—
119	—	—	—
127	.01	—	—
135	.02	—	—
147	.01	—	.05
157	.001	—	.02
171	.001	—	.01
186	.001	—	.01

*—Indicates no statistically significant difference.

tion; day 201, 231, and 261. In all 3 comparisons significantly more of the concurrent-stress animals had died. No other comparisons proved to be significant although the trend in all cases was in the expected direction, *i.e.*, stressed animals tended to die earlier than controls.

DISCUSSION

The results of this experiment indicate that the stress of enforced activity had a significant enhancing effect on the speed of development of methylcholanthrene-induced papillomas when activity stress was initiated concurrently with commencement of methylcholanthrene application. When this stress was initiated 15 days before application of the carcinogen, no statistically significant enhancement was observed though, as text-figure 1 indicates, this group showed consistently more papillomas throughout the experiment than was true of the control group.

Similarly the death rate showed that concurrent-stress animals died earlier than was true of either of the other 2 groups. Although they died earlier, the number of animals which had died when the experiment was terminated was equal for all 3 groups.

In a previous study (*12*) we reported on the effect of stress and gentling on epilation induced by painting with methylcholanthrene. In that study we found that a stressed group (5–10 electric shocks per day randomly

spaced over 8 hours) and a group of animals handled only during the application of methylcholanthrene and at times of hair-loss evaluation showed significantly less hair loss than a more frequently handled group painted with methylcholanthrene. Hair-loss evaluations were also conducted in the present experiment. We found that the control group developed such hair loss earlier and at a somewhat more rapid rate than the 2 experimental groups. These differences were, however, not statistically significant. No direct comparison between the earlier and the present experiments, in terms of hair loss, is possible because of differences in strain as well as differences in number of applications of the carcinogen. Hair losses in the present study occurred much earlier; after the fifth painting all animals had epilated areas of 1 cm.^{2} or larger, while in the earlier study a similar extent of hair loss did not occur in the control group until after the ninth application of the carcinogen.

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Chromosomal Studies on Primary Methylcholanthrene-Induced Sarcomas in the Mouse [1,2]

K. E. HELLSTRÖM,[3] *Institute for Tumor Biology, Karolinska Institutet Medical School, Stockholm, Sweden*

SUMMARY

Cytological studies of chromosomes have been made on 14 primary fibrosarcomas induced by intramuscular injection of methylcholanthrene. All tumors showed a considerable variability in chromosome number. Ten tumors were characterized by stem lines, which were changed chromosomally and were individually different for each neoplasm. These results agree with existing data on transplanted methylcholanthrene-induced sarcomas. Four tumors contained cells with the normal diploid number of 40 chromosomes as their dominating stem line and did not show any detectable structural modifications. The difference between tumors might be due to the fact that they have reached various stages of progression. It also appears that great structural or numerical chromosome changes are not necessary for the development of all tumors.—J. Nat. Cancer Inst. 23: 1019–1033, 1959.

PREVIOUS CYTOLOGICAL studies on mouse neoplasms have indicated that serially transplanted tumors, in general, and ascites tumors, in particular, represent heterogeneous cell populations, with chromosome numbers varying around a modal number which deviates from the normal euploid numbers of the host and is usually different for different tumors (*1*, *2*). This numerical variation has been attributed to various mitotic abnormalities, such as spindle multipolarity, c-mitosis, nondisjunction, and endomitosis (*1*). In addition, chromosome breaks and rearrangements may give rise to structurally changed "marker" chromosomes. Shifts in the chromosomal complement of tumor cells are often observed concomitantly with changes in different biologic characteristics such as the ability to grow in foreign genotypes or the sensitivity to antimetabolites (*3*). The cytogenetic instability of tumor cells has been contrasted with the high constancy of the chromosome complement now generally accepted for normal somatic cells (*4*, *5*).

Since the beginning of this century, when Boveri (*6*) proposed that malignant growth may be due to an unequal distribution of genetic

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material in the tumor cells, it has been frequently discussed whether chromosomal changes could be directly involved in the neoplastic transformation or represent a secondary consequence of tumor growth. In trying to elucidate this question it is essential to study primary neoplasms rather than serially transplanted tumor lines, to avoid possible complications due to the secondary evolution that long-propagated tumors are known to undergo. It seems to be necessary to investigate whether important general differences exist between the chromosome complements of primary and transplanted tumors, and whether all or only certain primary tumors are distinguishable from normal cells, chromosomally, etc. Only a few clear-cut results have been obtained with primary tumors until the present, owing to earlier technical difficulties with such material.

Most primary tumors hitherto studied have shown chromosomal changes with an increased variation of chromosome numbers as compared to normal somatic cells and, in most instances, with new, aneuploid modal numbers. Ford *et al.* (*2*) developed a satisfactory technique for solid tumors (*7*) and studied one spontaneous and many X-ray-induced primary reticular neoplasms of the mouse; most, but not all, tumors had a cytogenetic individuality, with aneuploid modal numbers that were different from the chromosome numbers of normal cells and often individually different for different tumors. Ising and Levan (*8*), Fritz-Niggli (*9*), and others have studied some human tumors with modal numbers that were aneuploid. Koller (*10*) noted a considerable variability in the chromosomal number of exudate cells from human tumors; most cells were diploid. However, Leuchtenberger, Leuchtenberger, and Davis (*11*) and Atkin and Richards (*12*) measured the DNA content of various human tumor cells whose values indicated mostly hyperdiploid and near-tetraploid chromosome numbers that were dissimilar for different tumors.

In other systems a different cytological picture has been observed. Tjio and Östergren (*13*) examined a number of primary mammary carcinomas of milk-agent-carrying mice and noted that, with little variation, the majority had a modal number of exactly 40 chromosomes, the diploid number of the mouse. One of the chromosomes was heteropyknotic in all tumors except 1, which had only 39 chromosomes. Bayreuther (*14*) studied a number of different virus-induced tumors in the mouse, rabbit, and fowl, and all tumors but 2, the Shope carcinoma and the V_{x2} Rous carcinoma, were indistinguishable from normal somatic cells with regard to their chromosome complement. He found a constant diploid chromosome number in mammary cancers of milk-agent-carrying mice even after a series of transplant generations. Normal diploid idiograms have also been found by Levan (*15*), for a virus-induced mouse leukemia, and by Gopal-Ayengar (*16*), for mammary cancer of the mouse.

Since methods are now available (*7*, *17*) that permit the analysis of solid tumors with almost the same exactness as with ascites cells, it seemed to be of interest to examine other types of primary tumors. This

paper reports results on a series of methylcholanthrene-induced fibrosarcomas in the mouse. Available chromosome data on material of this type have been limited to transplanted tumors, and show that different transfer lines are characterized by a cytogenetic individuality (*17*, *18*). The purpose of our study was to find out whether this individuality exists at the primary stage or arises later in connection with serial transfer.

MATERIALS AND METHODS

The tumors studied were induced in different F_1 hybrid mice: namely, A/Sn × A.CA; A.BY × A.CA; A/Sn × A.SW; A.CA × A.SW (*19*); and DBA/2 × C57BL. All strains had been maintained by continuous single-line brother × sister mating.

The mice were kept on a standard diet in pellet form, with drinking water and pellets available *ad libitum*. Tumors were induced in animals of both sexes at the age of 1 to 2 months by an injection of 0.5 or 0.05 mg. of methylcholanthrene in 0.1 ml. trioctanoin into the musculature of the right thigh.

Fourteen tumors which appeared 3 to 6 months after the injection were used for cytological analyses. They were fixed about 3 weeks after their appearance and were then of about the same size. Some biologic data for the tumors are given in table 1.

Cytological methods.—A slight modification of the techniques described by Ford and Hamerton (*7*) and Bayreuther and E. Klein (*17*) was used. Mice were given 2 intraperitoneal injections of 0.04 percent Colcemid (Ciba) solution in doses of 0.1 ml. per 10 gm. of body weight, with an interval of 60 minutes, and killed by cervical dislocation 60 minutes after the second injection. The tumors were dissected out, care being taken to discard all necrotic parts, and small pieces of tumors were prepared by mincing them with scissors. The material was incubated in a 1.12 percent sodium citrate solution for 15 to 25 minutes at 37° C., fixed in chilled acetic acid-alcohol, 1:3, for 1 to 2 hours, transferred to 30 percent alcohol for 3 to 5 minutes and to distilled water for 3 to 5 minutes, and then hydrolyzed in 1 N HCl at 60° C. for 8 minutes. After chilling, the hydrolyzed material was stained in Feulgen reagent for 1 to 2 hours, transferred to SO_2 water for 3 minutes, and then stored in 45 percent acetic acid in a deep-freeze unit, at about −15° C. (*20*), in which the material can be stored for many months without changes in quality. Squash preparations were made in the usual way with siliconized coverglasses.

The squash preparations were studied with a Zeiss Standard phase-contrast microscope. Cells suitable for chromosome counting were selected at a magnification of 375× and were analyzed at 1500×. Only metaphase figures with well-dispersed chromosomes were examined. Methylcholanthrene-induced fibrosarcomas represent a comparatively difficult material for cytological technique, and a certain proportion of the

metaphases had to be discarded because of insufficient dispersion of the chromosomes.

The most serious error with the squash technique is represented by the loss of chromosomes. To avoid it, all cells selected for analysis had intact outlines, were free from other mitotic cells, and were scanned carefully for the possible presence of free chromosomes in their neighborhood. The frequency of obviously damaged cells was only about 5 percent in the present material. This makes it improbable that squashing errors could seriously interfere with the results.

All chromosome counts were made on drawings prepared with an Abbe's counting apparatus, which permitted a fairly exact count. Repeat counts of the same cell gave identical numbers in about 95 percent, while in about 5 percent of the countings there was a variation of ± 1 chromosome.

Since primary methylcholanthrene-induced sarcomas contain comparatively few mitotic figures, the number of cells analyzed for each tumor was between 20 and 52. Small differences could be found in chromosome numbers when two samples of 20 cells were counted from the same tumor and compared; this can be attributed to biologic rather than methodological variation. However, the degree of variability and the modal chromosome number were about the same in repeat counts.

RESULTS

Sampling data for the different tumors are presented in table 1. The findings are only summarized, since the chromosome numbers of tumors 1 to 14 are shown in detail in table 2. Drawings of representative metaphases from each sarcoma are presented in figures 1 to 3.

The data show that the tumors studied represent a cytogenetically heterogeneous group. Individual tumors were different with regard to their modal chromosome numbers and the degree of numerical variation. Four tumors had, predominantly, cells with an apparently normal chromo-

TABLE 1.—Some biologic data for the 14 primary sarcomas investigated

Tumor No.	Genotype (F_1 *hybrids*)	Sex	Dose of methylcholanthrene (*mg.*)
1	A/Sn × A.SW	♂	0.5
2	A/Sn × A.SW	♂	0.5
3	DBA/2 × C57BL	♀	0.05
4	A.SW × A.CA	♂	0.5
5	A/Sn × A.SW	♂	0.5
6	A/Sn × A.CA	♂	0.5
7	A/Sn × A.SW	♂	0.5
8	A/Sn × A.SW	♂	0.5
9	A.BY × A.CA	♂	0.5
10	A/Sn × A.SW	♂	0.5
11	A/Sn × A.CA	♀	0.5
12	A/Sn × A.CA	♀	0.5
13	A/Sn × A.SW	♂	0.5
14	DBA/2 × C57BL	♀	0.05

TABLE 2.—Distribution of cells with different chromosome numbers in the 14 primary sarcomas investigated

| Tumor No. | Number of chromosomes | | |

some complement of 40 chromosomes, while 10 tumors had most of their cells chromosomally differing from normal somatic cells.

Chromosomal variability between different tumors was remarkable. Some tumors (*e.g.*, #3, 5, and 13) displayed an extensive variability without any dominating chromosome numbers. One tumor (#13) showed a variation between 42 and 61 chromosomes, most of the cells being hyperdiploid. In contrast, other tumors were characterized by a small variability. In tumor 11, for instance, 27 of 50 cells had 40 chromosomes and the other 9 cells within the diploid range varied between 41 and 43 chromosomes.

Since no transplantations have been done from the primary tumors studied, it is difficult to decide which cells correspond to the stem lines responsible for the reproduction of the tumors. For the sake of convenience the term "stem line" has been used to denote the group of cells having the modal chromosome number of the tumor. Some tumors had one dominating stem line; in 1 tumor (#8) 21 of 23 cells were diploid or near diploid, whereas the 2 other cells counted seemed to represent a tetraploid stem line. In other tumors, 3 stemline cells were present, and in 1 tumor (#7) 4 groups of cells occurred together, each represented in approximately the same frequency.

Similar to findings on other populations of neoplastic cells, most tumors had predominantly diploid or hyperdiploid stem lines (#1, 3, 4, 5, 6, 8, 9, 10, 11, and 13). Two tumors had about the same number of cells in the hyperdiploid and the hypertetraploid stem lines (#2 and 12) and 1 tumor had its stemline cell in the hypertriploid range (#14). In most instances the stem lines had aneuploid chromosome numbers, but 5 tumors (#4, 8, 10, 11, and 12) had mainly 40 chromosomes. One of these tumors (#4 and fig. 1E) contained, as its longest chromosome, one metacentric marker and is therefore not truly diploid. The other 4 tumors (figs. 2, C, E, and F, fig. 3A) had 40 chromosomes without detectable structural modifications. Drawings of metaphases from spermatogonia were compared with drawings from these latter tumor cells and there were no apparent differences in the structure or size of chromosomes. However, modifications of the chromosomal structure can very well occur without being detected, since mouse chromosomes are poorly differentiated. The only tumor with a typical marker chromosome was number 4 (fig. 1E).

The material was too small to permit any conclusions about possible correlations between the dose of methylcholanthrene injected and the chromosomal complement. However, both tumors induced with the small dose were aneuploid (#3 and 14) indicating that the changed chromosome numbers in most tumors were not due to the high dose of methylcholanthrene used with the other tumors.

DISCUSSION

Increased knowledge of the chromosomes of different primary tumors is important for a better understanding of the genetics of cancer cells.

Certain information about chromosome number and structure can be obtained by cytological methods. It must be realized, however, that such methods are crude and only permit the detection of gross changes, which constitute merely a limited part of all possible genotypic changes. For this reason, it is more difficult to interpret findings of apprently normal chromosome complements than observations of changed chromosome sets with abnormal numbers and structurally altered marker chromosomes.

The group of primary methylcholanthrene-induced sarcomas studied in this investigation was cytogenetically heterogeneous, with mostly aneuploid stem lines in the hyperdiploid, triploid, or hypertetraploid region, and with a variability of the chromosome number in all tumors. This is in contrast to the constant chromosome numbers and the low variability in normal somatic cells (*4*, *5*). The data obtained are similar to the results of Ford *et al.* (*2*), Ising and Levan (*8*), and Fritz-Niggli (*9*), who have studied different spontaneous or induced primary tumors. Their results seem to differ from the data on some tumor of known viral etiology obtained by Tjio and Östergren (*13*), Bayreuther (*14*), Levan (*15*), and Gopal-Ayengar (*16*), which all show considerably less variability and predominance of diploid cells.

The cytogenetic individuality noted in most methylcholanthrene-induced tumors is reminiscent of differences in their biologic behavior. A biologic individuality for different mouse sarcomas has been reported by Klein and Klein (*21*), who studied the behavior of mouse sarcomas induced in F_1 hybrid mice, between two isogenic resistant mouse strains, by the same dose of methylcholanthrene. The tumors gave rise to variant sublines selectively compatible with one or the other of the parental strains in a frequency which was characteristic for each individual tumor.

Four of the 14 sarcomas that we studied seemed to contain the normal diploid mouse chromosome complement, 40, as their stem line, although they showed a considerable variation in the hyperdiploid region. It cannot be determined with present cytological technique whether structural changes of the chromosomes had occurred in such apparently normal cells. Levan (*1*) has shown that such rearrangements of the entire chromosome complement are important in the evolution of mouse tumors. In any case, there seems to be a certain difference between the four "normal" tumors and the rest of the material that shows chromosome changes. This difference may be due to the fact that the tumors have reached different stages of progression. Deviating cells potentially capable of forming new stem lines have been found in all 4 "diploid" tumors.

It is possible that cancer is a result of the interaction of many kinds of independent changes in cells, which are different for different tumors. In spite of the fact that chromosome changes are visible in most primary tumors the investigation suggests that great structural or numerical chromosome changes are not necessary for the development of all tumors, even if exception is made of the apparently diploid virus tumors described by Tjio and Östergren (*13*), Bayreuther (*14*), and others. More knowl-

edge about different types of primary tumors is necessary before definite conclusions can be drawn about the correlations between tumor development and chromosome rearrangements.

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PLATES

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PLATE 94

FIGURE 1.—A: Metaphase of stemline cell of tumor 1; 42 chromosomes. B: Metaphase of stemline cell of tumor 2; 84 chromosomes. C: Metaphase of stemline $_1$ cell of tumor 3; 43 chromosomes. D: Metaphase of stemline $_2$ cell of tumor 3; 52 chromosomes. E: Metaphase of stemline of tumor 4; 40 chromosomes including one metacentric marker. F: Metaphase of stemline cell of tumor 5; 41 chromosomes. × 1,150

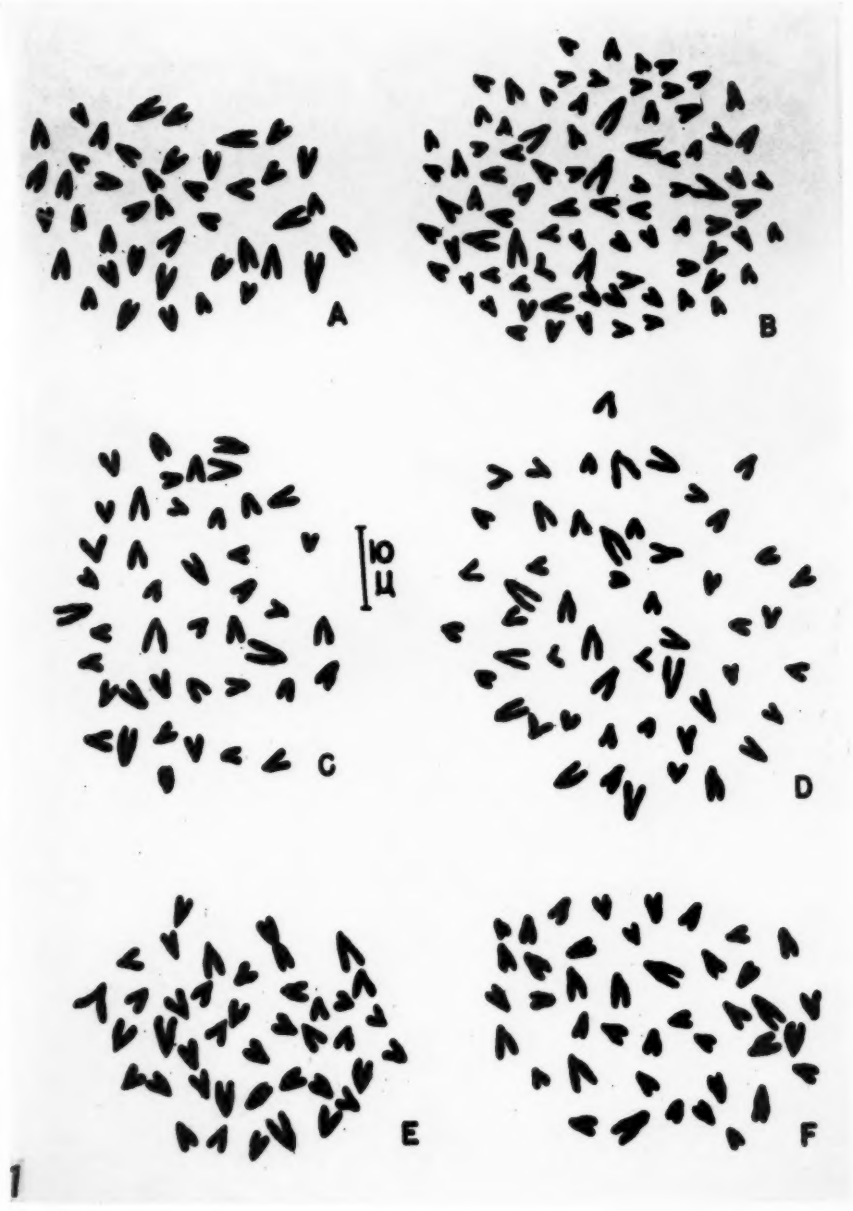
A
B
10 μ
C
D
E
F

PLATE 95

FIGURE 2.—A: Metaphase of stemline cell of tumor 6; 42 chromosomes. B: Metaphase of stemline cell of tumor 7; 40 chromosomes. C: Metaphase of stemline cell of tumor 8; 40 chromosomes. D: Metaphase of stemline cell of tumor 9; 43 chromosomes. E: Metaphase of stemline cell of tumor 10; 40 chromosomes. F: Metaphase of stemline cell of tumor 11; 40 chromosomes. × 1,150

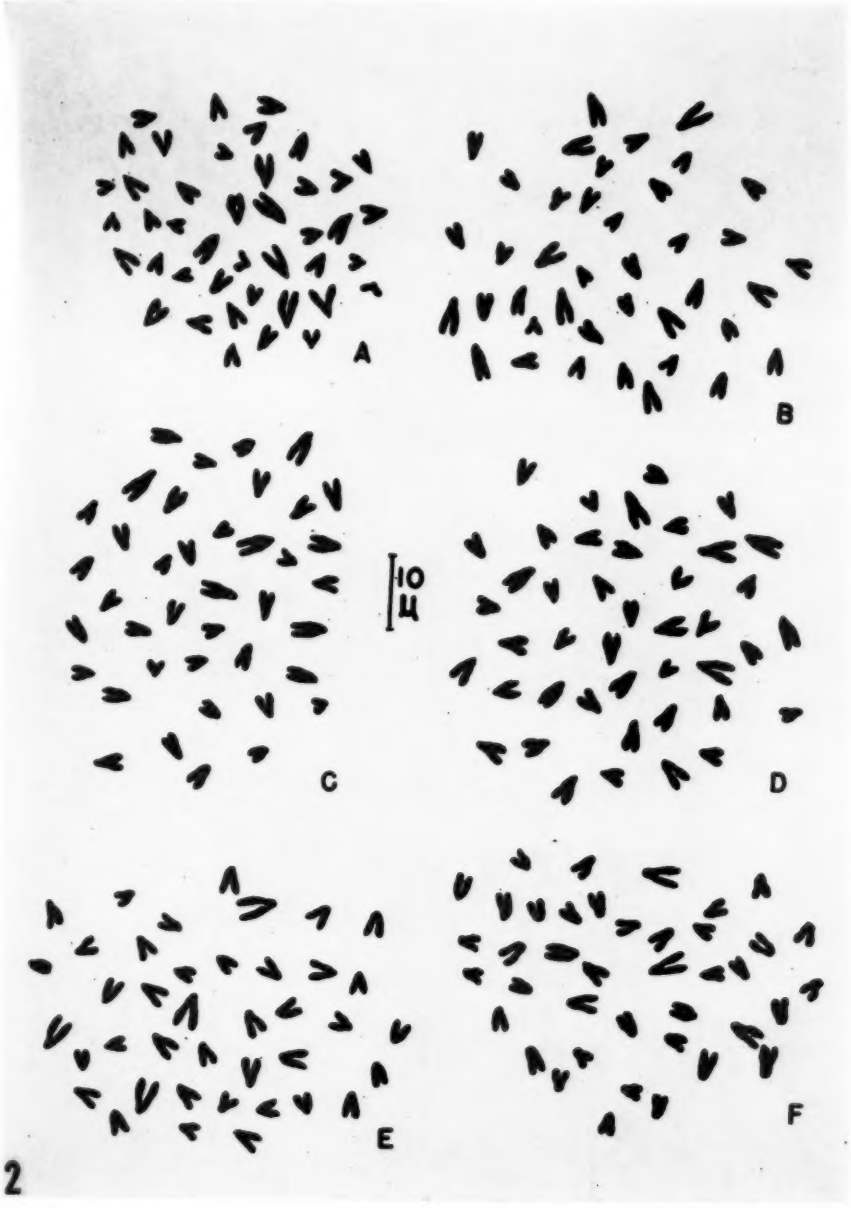
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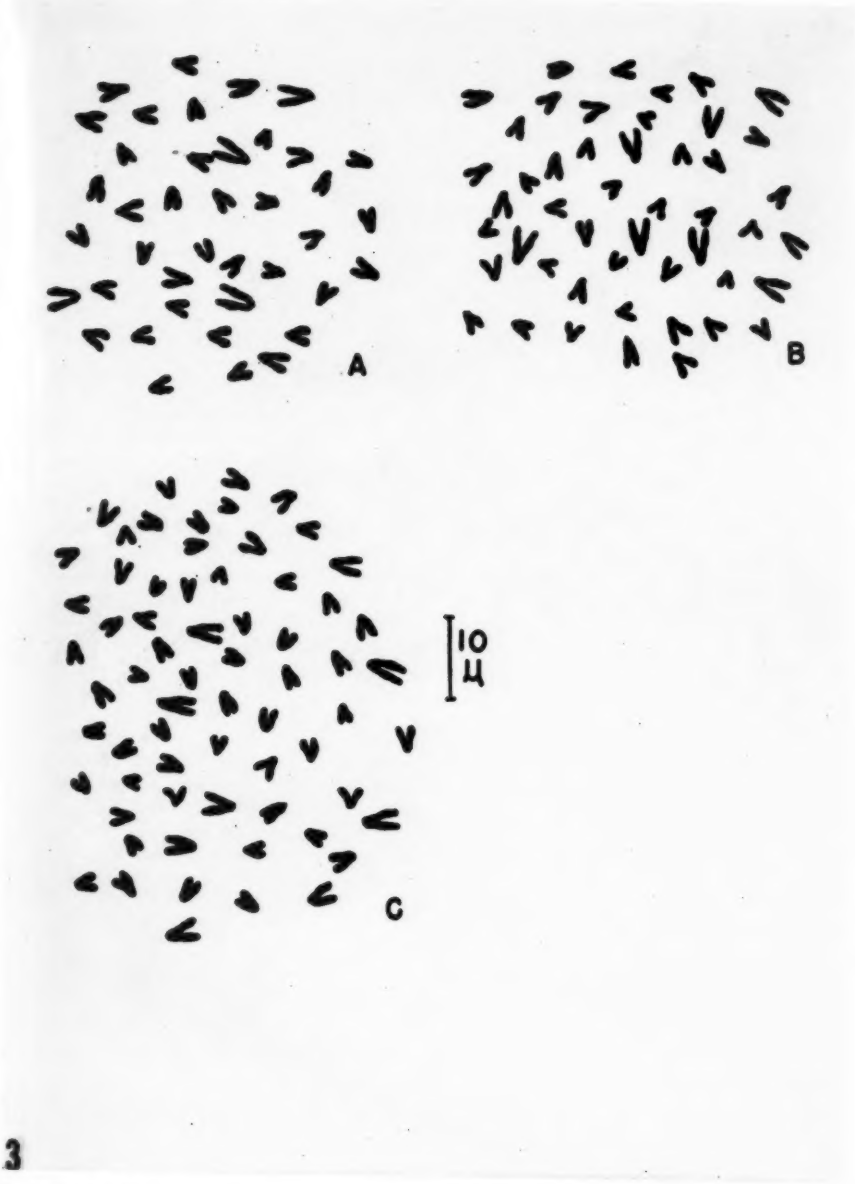
PLATE 96

FIGURE 3.—A: Metaphase of stemline cell of tumor 12; 40 chromosomes. B: Metaphase of stemline cell of tumor 13; 48 chromosomes. C: Metaphase of stemline cell of tumor 14; 66 chromosomes. × 1,150



3

Clonal Analysis of Variant Cell Lines Transformed to Malignant Cells in Tissue Culture [1]

KATHERINE K. SANFORD, RUTH M. MERWIN, GWENDOLYN L. HOBBS, JAMES M. YOUNG,[2] *and* WILTON R. EARLE, *Laboratory of Biology, National Cancer Institute,*[3] *Bethesda, Maryland*

SUMMARY

The following alternative interpretations of the difference between a low and high sarcoma-producing line of mouse cells derived *in vitro* from one cell of normal tissue origin have been examined: (1) The two lines might differ quantitatively in the proportion of malignant transplantable cells present in the cell populations. (2) Cells of the low line as compared with those of the high might possess entirely different heritable characteristics accounting for the low incidence of takes and the prolonged latent period for tumor development *in vivo*. A clonal analysis of the two populations has furnished evidence for the second interpretation. Five single cells from the low and high lines duplicated in their derived clones most of the characteristic properties of the line from which each originated, including the characteristic rate of tumor formation *in vivo*. Some differences between clones from the same line were also observed, revealing variations or potentialities for further change among individual components of the cell population. In addition, the type of culture medium was an important factor influencing the transplantability of tissue-culture cells to animals of the inbred strain of origin. The importance of transplantation for detecting the malignant conversion of cells *in vitro*, factors influencing the transplantability of tissue-culture cells, possible causes of the malignant transformation of cells *in vitro*, and interpretations of the differences between these cell lines and clones are discussed.—J. Nat. Cancer Inst. 23: 1035–1059, 1959.

PREVIOUS REPORTS from this laboratory (*1–3*) describe variant [4] lines of mouse cells derived *in vitro* from one cell originating from normal tissue. The cell lines were separated as multicellular populations 2 months after single-cell isolation, and two of the lines were continued in culture for 7 years. During the first year, these lines acquired divergent characteristics, both morphologic and metabolic. When implanted into

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[2] Tissue Bank, Naval Medical School, National Naval Medical Center, Bethesda, Maryland.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[4] The term "variant" rather than "mutant" has been used in this paper, since the term "mutant" strictly implies a gene mutation, which at the present time cannot be detected in asexually dividing mammalian somatic cells. Although the mechanism of change in these clones may have been genic in origin, the possibility of chromosomal aberrations, mitochondrial alterations, or changes in other replicating cytoplasmic or nuclear determinants cannot be eliminated at the present stage of our knowledge.

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mice of the inbred strain of origin, cells from one line grew as sarcomas in 97 percent of the animals, and the other line grew in 1 percent. The two lines were designated as the high and low sarcoma-producing lines or "high" and "low" lines, respectively. If cells from the low line were implanted into X-irradiated mice, a larger number of mice developed tumors, each tumor appearing after a long latent period (*2*).[5]

The characteristics of the two lines remained fairly stable during the years of culture (*2*), though occasionally a low-line culture resembled one from the high line or produced tumors morphologically similar to those arising *in vivo* from cells of the high line. From two such tumors, cell strains were isolated in culture that exhibited all the known morphologic, metabolic, and physiologic properties of high-line cells (*4*).

In an effort to interpret the difference between the two lines in tumor-producing capacity, the following questions have been considered in this study: (1) Did the low line produce a few tumors with prolonged latent periods because the cell population contained a smaller proportion of malignant, transplantable cells? These malignant cells might be similar to those of the high line or might be altered morphologically since low- and high-line tumors generally differed in morphology (*1*, *2*). (2) Did cells of the low line, as compared with those of the high, possess entirely different heritable characteristics that accounted for the low incidence of takes and prolonged latent period for tumor development *in vivo*? In order to answer these questions, a clonal analysis of the two populations was undertaken.

This paper reports the establishment of five clones, one from the high line and four from the low, and compares the characteristics of the clones with each other and with the original lines from which they were derived. Investigations of the chromosomal constitution of the cells and of their rates of anaerobic glycolysis *in vitro* were carried out by Chu and associates (*5*) and Woods and associates (*6*), respectively. Four of the clones were also examined by Scott and associates for activities of certain of the glycolytic enzymes (*7*).

In order to establish the clones, it was necessary to change the type of culture medium. The original medium, a mixture of horse serum, chick-embryo extract, and balanced saline, failed to support growth and proliferation of single, isolated cells. When replaced with chemically defined medium NCTC 109 (*8*, *9*) supplemented with 10 percent horse serum, clones were readily obtained. The parent high and low lines as well as the clones were cultured in the new medium for comparative purposes. Culture in this medium influenced the tumor-producing capacity of the cells. Cells of the original "low" line now grew progressively in almost every mouse injected. However, cells of the two lines still differed significantly in their rates of growth to form palpable tumors when implanted in mice. Cells of the original "low" line still exhibited a significantly longer latent period for tumor formation than cells of the high line.

[5] The tumor latent period measured the interval from injection of cells to the appearance at the injection site of a palpable tumor that continued to grow progressively and was ultimately diagnosed as a sarcoma.

Although the type of culture medium was found to alter the transplantability of the cells, the original two cell lines will still be referred to in this paper as the "high" and "low" lines [6] in order to simplify the presentation.

Since all clones from the low line produced tumors with typically long latent periods—significantly longer than those exhibited by high-line cells—it was concluded that the low line, rather than containing a smaller proportion of malignant, transplantable cells, possessed different heritable characteristics, including the slow rate of growth to form tumors *in vivo*. This heritable difference between the two lines is probably related to different metabolic properties and possibly stems from heritable differences in enzyme activities, as will be discussed later in this paper.

MATERIALS AND METHODS

Culture procedure.—Cells were cultured on the glass floor of the flask in either of the following two types of culture fluid: (1) A mixture of 40 percent horse serum, 20 percent chick-embryo extract (1:1.25), and 40 percent Earle's saline prepared as described previously (*10*). This medium will be referred to as the "stock medium." (2) Chemically defined medium NCTC 109 (*8*, *9*) in which a portion of the saline was replaced by horse serum to a final concentration of 10 percent. This medium will be referred to as the "experimental medium." The volume of culture fluid used for each culture depended on the density of cell growth, ranging from 1 to 2 ml. in Carrel D-3.5 flasks and from 8 to 15 ml. in T-60 flasks (*11*). The fluid medium was renewed thrice weekly, at which time each culture was gassed with a humidified mixture of 5 percent CO_2 in air to adjust the *p*H, and each flask was sealed with a silicone stopper. Cells were subcultured when the cell sheet covered the floor of the flask, usually at 1- to 2-week intervals. In subculturing, the cells were shaken from the substrate, and the cell suspension was transferred by pipette to two or three flasks.

Procedure for single cell isolation and growth.—Approximately 6 to 24 hours before single-cell isolation, the stock medium on the culture was withdrawn and replaced with an equal volume of experimental medium. At the time of single-cell isolation, cells were shaken from the floor of the culture flask, and the resulting cell suspension was diluted and passed through a double-platinum sieve (*12*). The sieved cell suspension was drawn freehand, without use of a dissecting microscope, into capillary pipettes which were sealed, examined, and planted according to procedures described previously (*13*). Each capillary segment was planted in a thin film of plasma clot, just sufficient to anchor the capillary to the glass floor of the Carrel flask. All solutions including the chicken plasma had been filtered through an 03 porosity Selas filter.

[6] The low and high lines were originally referred to as culture lines of substrains III and VII, respectively (*1*). As carried in this laboratory these lines are designated NCTC strains 2049 and 1742.

Photographic records.—Randomly selected cultures were photographed at intervals by means of photomicrographic procedures previously described (*14*). Cultures were photographed 24 hours after a renewal of culture fluid.

Procedure for measuring population increase in vitro.—In order to ascertain the rate of population increase *in vitro*, replicate cultures were prepared from sieved and stirred cell suspensions according to methods previously described *(12)*. At the time of inoculation, and at daily intervals thereafter, cultures were selected at random for the enumeration of cell nuclei (*15*).

Procedure for assay of tumor-producing capacity.—By means of aseptic techniques (*16–18*), cells were injected intramuscularly as a suspension in 1 ml. of culture fluid into the left thigh of 1- to 4-month-old male mice of strain C3H/HeN. The inoculum size was estimated from the enumeration of cell nuclei in two 0.2-ml. aliquots of the agitated suspension, taken at the beginning and end of the injections (*15*). Some of the mice were X-irradiated with a whole-body dose of 425 roentgens before injection.[7] The general procedure in the immunization experiments has been described (*2*). All mice were observed for 3 to 4 months after injection. In order to ascertain the tumor latent period, each mouse was examined weekly for any palpable growth at the injection site. Autopsies were carried out on all mice. Strips of tumor tissue were fixed in formalin-Zenker, sectioned, and stained with hematoxylin and eosin and with van Gieson's picrofuchsin. Diagnosis of sarcoma was made by microscopic examination of the tumor sections.

Procedure for studying growth and vascularization of cells implanted in living mice.—The growth and vascularization of the cells implanted subcutaneously were studied in the living mouse by means of a transparent-chamber technique developed by Algire and Legallais, and modified by Merwin to facilitate observations of tissue-culture cells (*19*, *2*).

EXPERIMENTS AND RESULTS

Origin of Clones and Cell Strains

Four clones were established from the low line and one from the high line. Two clones from the low line, designated NCTC clones 2445 (fig. 1) and 2470, were originated on May 15, 1956, approximately 6 years after the origin of this line *in vitro*. An additional clone from the low line, NCTC clone 2555, was started on September 11, 1956, from a cell, the nucleus of which divided to form a binucleate cell. It appeared that the two nuclei of the cell then fused giving rise to a single large nucleus. This clone grew slowly and required 7 months to fill a D-3.5 Carrel flask as compared with 2 to 3 months for all other clones. A fourth clone,

[7] The authors express their appreciation to Mr. Henry Meyer of the Radiation Branch of this Institute, who irradiated the mice according to methods previously described (*2*).

NCTC clone 2426, was originated on April 24, 1956, from a cell isolated from a 25-day-old culture of sarcoma tissue. The sarcoma arose in an irradiated hybrid mouse (BALB/c × C3H)F_1 from an injection of low-line cells. Cells of the clone grew progressively in C3H mice and therefore could not have been derived from a cell of the hybrid host (*20*). The one clone from the high line, NCTC clone 2472, was started on May 15, 1956.

Some cultures of high and low lines were transferred to the experimental medium on November 26, 1956.[8]

Characteristics of High- and Low-Line Cells and of Clones in Stock and Experimental Media

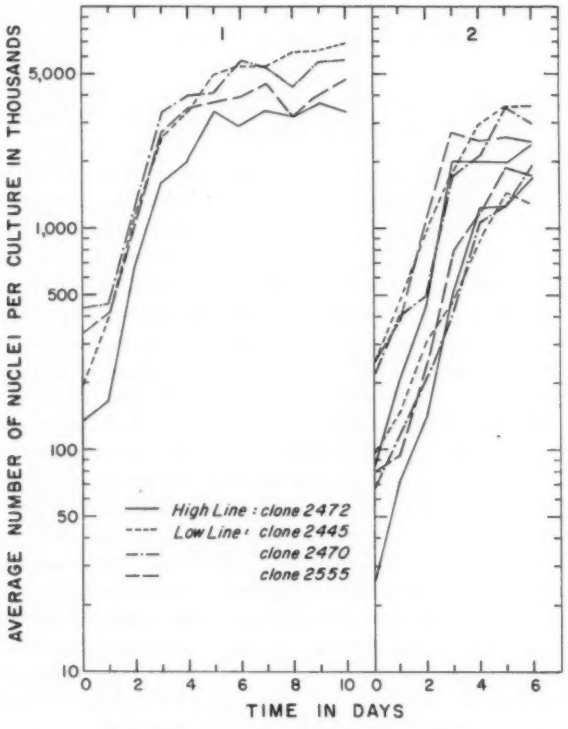
Cell morphology and growth pattern in vitro *and* in vivo.—The morphology of each clone *in vitro* resembled that of the parent line from which the clone was derived (figs. 2–8). A characteristic of the low line and its clones as compared with the high line and its clone was the frequent appearance of large mononuclear and polynuclear giant cells (figs. 4 and 9–11). These cells appeared in dense cultures when grown on stock or experimental medium. Tumors arising from low-line clones were uniformly soft and hemorrhagic; tumors from the high-line clone were solid and firm, and the microscopic patterns were different, as described previously for the original lines (*1*). Cells of both lines cultured in experimental medium exhibited more variation in cell morphology and growth pattern, both *in vitro* and *in vivo*, than cells grown in stock medium. However, the clones grown on experimental medium showed less morphologic variability.

Rate of population increase of clones in vitro.—There appeared to be no conspicuous difference between the three low-line clones (2445, 2470, and 2555), and the high-line clone (2472) with respect to their rates of population increase when grown in the experimental medium (text-fig. 1). Similarly, in the previous studies (*2*) the two parent lines exhibited approximately the same proliferation rates when cultured in stock medium.

Capacity of lines and clones to produce sarcomas in mice of the strain of origin.—Experiments testing the influence of the two types of culture medium on tumor-producing capacity of low- and high-line cells are summarized in text-figure 2. During the first 10 days of growth in the experimental medium, the tumor-producing capacity of the cells was indistinguishable from that of the cells in stock medium. However, by 51 days and particularly after 108 to 150 days of culture a significant increase in sarcoma-producing capacity was observed. Although the incidence of tumors from low-line cells was increased and the tumor latent periods were reduced, there was still a significant difference between cells of the two lines with respect to the rate at which they formed palpable

[8] As carried in this laboratory these high- and low-line cells cultured in experimental medium are designated NCTC strains 2672 and 2673, respectively.

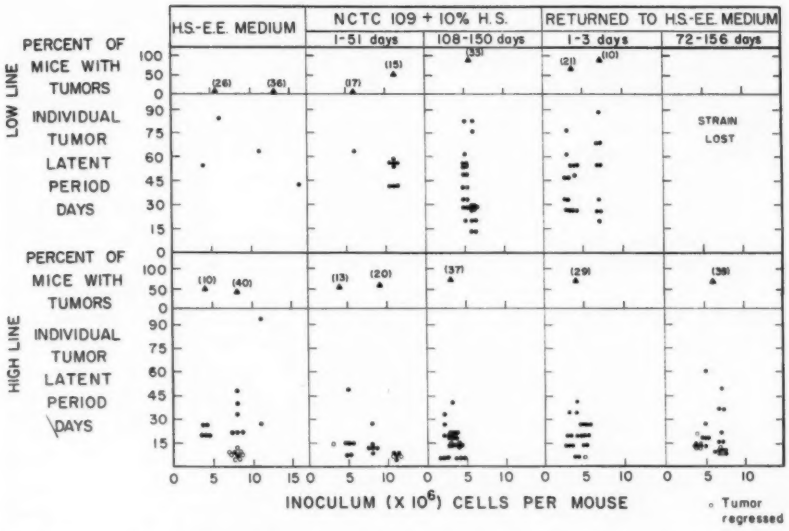
tumors in mice. The increase in tumor-producing capacity was not abolished by returning the cells to stock medium for 1 to 3 days. After the 3-day period, low-line cells did not continue to grow. The high line maintained the high tumor-producing capacity for the duration of the experiment, 156 days.



TEXT-FIGURE 1.—Results of experiments (1 and 2) comparing rates of population increase of clones at different inoculum sizes in medium NCTC 109 supplemented with 10 percent horse serum. The average number of cell nuclei per culture is plotted on semilogarithmic grid against time in days. Each determination represents the average of 2 cultures.

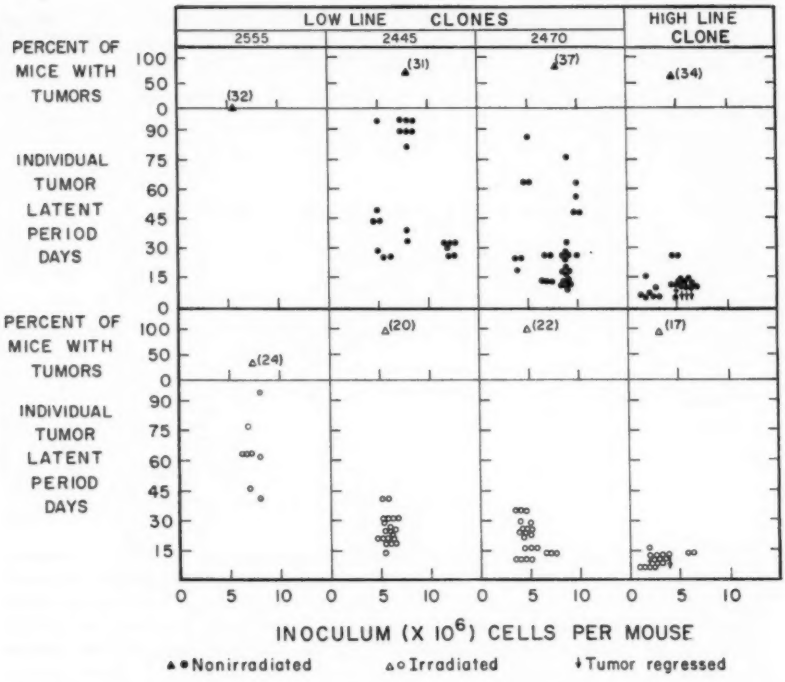
Thus, culture in the experimental medium significantly increased the incidence of takes from cells of both lines. The change occurred gradually, was not readily reversed on return to stock medium, and did not obliterate the difference between the two lines in their rates of tumor formation.

The results of assaying the three low-line clones (2445, 2470, and 2555) and the high-line clone (2472) in nonirradiated and X-irradiated mice of strain C3H are summarized in text-figure 3.



TEXT-FIGURE 2.—Influence of serum-supplemented medium NCTC 109 on sarcoma-producing capacity of high- and low-line cells. *Upper panel* shows results of injecting low-line cells and *lower panel* results of injecting high-line cells into strain C3H mice. Cells had been cultured (1) continuously in stock medium (H.S.-E.E. medium), (2) in experimental medium (NCTC 109 and 10% H.S.) for the periods indicated, or (3) in experimental medium for 4 to 9 months with return to the original stock medium for the days indicated. *Upper section* of each panel shows the percentage of mice developing tumors plotted against mean inoculum received by mice in the group. With one exception, mice were grouped into those receiving 7 million cells or less and those receiving more than 7 million cells per mouse. The one exception is the group of low-line cells returned to H.S.-E.E. medium in which the mice were grouped into those receiving 7 million cells and those receiving less than 7 million cells per mouse. Numbers in parentheses indicate numbers of animals injected. *Lower section* of each panel presents the individual tumor latent periods plotted against inoculum size. A tumor that regressed is indicated by an open circle.

Clone 2555, which arose from a binucleate cell, differed from the other two low-line clones in failing to grow progressively in any nonirradiated mice. After a prolonged latent period, the cells gave rise to a few tumors in irradiated hosts only. The other two low-line clones, 2445 and 2470, produced a high incidence of sarcomas in nonirradiated and irradiated hosts. A slight difference between the two clones with respect to tumor latent periods was apparent. The fourth low-line clone, 2426, isolated after mouse passage also produced a high incidence of sarcomas. When tested after 14 months *in vitro* following mouse passage, cells of this clone produced tumors in 16 of 20 mice (80%) injected with 11×10^6 cells per mouse. Tumor latent periods averaged 35 days (range 8 to 100). Thus three of the clones resembled the original line cultured in experimental medium, and one clone gave rise to a lower incidence of tumors.



TEXT-FIGURE 3.—Sarcoma-producing capacity of clones as assayed in strain C3H mice. *Upper panel* presents results of assays in nonirradiated and *lower panel* assays in X-irradiated mice. For description of chart, *see* legend text-figure 2. Percentage of mice developing tumors was plotted against mean inoculum received by all mice injected. A tumor that regressed is indicated by an *arrow*.

The high-line clone resembled the original line cultured in experimental medium. A large number of tumors developed with some tendency toward regression. Tumor latent periods were significantly shorter than observed with any of the clones from the low line, in comparisons between mice receiving equal numbers of cells.

Capacity to initiate a vascular response in the host.—Cells of the five clones and of the high and low lines in experimental medium were implanted in transparent chambers in strain C3H mice for observation of their growth and vascularization in the living host. The following observations were based on a study of 27 mice, 3 to 6 of which were implanted with each clone or strain, at inoculum levels ranging from 0.3 to 9 $\times$ 10^5 cells per mouse.

Previous studies (*2*) showed that high-line cells in stock medium implanted in transparent chambers induced a vascular reaction characterized by an enlargement and convolution in the blood vessels of the host by the 4th day after injection. The implant became vascularized and

formed small tumors, which regularly regressed between 11 and 25 days of growth. In the present study, the high line and the derivative clone 2472 in experimental medium induced a similar reaction in the host vessels. The cell mass was vascularized between 7 and 11 days, but only 1 implant of the 6 regressed in 11 to 25 days. The others regressed later or were still growing at 54 days after injection when the mice were killed. It is of interest to note that high-line cells in experimental medium, implanted into 2 mice previously immunized by the same cell strain, elicited a weak vascular reaction in the host and disappeared thereafter.

In previous observations (*2*) low-line cells in stock medium caused no host vascular reaction, though some implants of cells became vascularized, but all evidence of the implanted cells was gone by 10 to 30 days. In the present study, low-line cells in experimental medium and three of the low-line clones occasionally initiated a delayed and weak vascular reaction at 11 days. The implants were vascularized by 18 days and continued to grow. Although 5 of the 10 observed for a long time regressed by 34 days, 5 continued to grow progressively. However, implants of the fourth clone (2555) failed to elicit any vascular reaction and disappeared by 11 days.

Influence of culture medium on antigenicity of cells.—Previous experiments (*2*) demonstrated that high- and low-line cells in stock medium injected into mice of the strain of origin (C3H) induced immunity to a second implant of cells of the same type. This immunity was shown to develop specifically from the injected cells and not from the culture medium. Further, the two cell lines were shown to cross-immunize C3H mice. The question arose whether the increased transplantability of the cells brought about by culture in experimental medium might result from a loss of antigenicity of the cells for C3H mice.

The results of 4 experiments testing this possibility are summarized in table 1. In the first 3 experiments, mice were injected subcutaneously (in leg or tail) with suspensions of cells grown for 7 months to over 2 years in the experimental medium. In the last experiment, mice were inoculated in the tail with tumor tissue developed in strain C3H mice from cells cultured for over 2 years in experimental medium. Two to 3 weeks after injection, the injected mice and noninjected control mice of the same age were challenged by injecting 1 ml. of cell suspension into the thigh muscles. Cells used for challenge had been cultured in experimental medium or in stock medium, as indicated in table 1. In the first 2 experiments mice were X-irradiated before challenge, since cells in stock medium often fail to grow in nonirradiated hosts (*1*, *2*).

The results showed clearly that high- and low-line cells cultured for long periods in experimental medium still induced immunity when injected into mice of the strain of origin. The first injection of cells almost completely blocked the development of tumors from cells of the second injection, unless an excessively large inoculum of cells was used for challenge. Under these conditions (expts. 3 and 4), tumors developed but at a significantly slower rate than in the nonimmunized controls.

TABLE 1.—Growth of tumors in mice that had received an injection of high- or low-line cells as compared with tumor growth in untreated mice

Experiment No.	Cells used for first injection	Cells used for challenge	Challenge inoculum ($\times 10^6$ cells per mouse)	Results: Mice with tumors / Number of mice challenged — Injected	Results: Mice with tumors / Number of mice challenged — Noninjected	Average tumor latent period (days) — Injected / Noninjected
1.	High line in expt. medium	High line, expt. medium	2.9	1/5*	2/2	—
		High line, stock medium	1.8	0/4*	2/3†	—
2.	Low line in expt. medium	Low line, expt. medium	0.9	0/2	2/2	—
		Low line, stock medium	5.6	0/2‡	2/2	—
3.	Low-line clone in expt. med.	Low-line clone in expt. medium	14.1	3/5*	5/5	59/13
4.	Tumor from low-line clone	Low-line clone in expt. medium	13.4	9/10*	5/5	41/14

*Tumor developed at site of first injection as follows: Experiment 1, in 2 mice challenged with cells in experimental medium and 1 mouse challenged with cells in stock medium; experiment 3, in 3 mice; experiment 4, in 4 mice.

†Additional mouse developed a tumor that regressed.

‡Area of cells 2 mm. in diameter found in 1 mouse at autopsy, 117 days after challenge.

DISCUSSION

One of the problems in studying the malignant transformation of normal tissue cells *in vitro* is to detect the change in the cells. In the frequent reports or discussions of such transformations in the literature, many different criteria for malignant change have been used or suggested (*21–32*). Since it is generally accepted that a malignant tumor is an autonomous, progressively growing neoplasm, and until some property of cells in culture can be definitely correlated with this capacity, only one test for malignant change in tissue-culture cells is available. This test is to assay the cells for their capacity to grow autonomously and progressively in animals of the strain of origin. For such a test, factors influencing the transplantation of tissue-culture cells assume considerable importance. A few of these will be considered here.

One factor is the development of immunogenetic differences between cultured cells and host. All studies from this laboratory on the malignant transformation of cells *in vitro* have been carried out on tissue cells from inbred strains of animals in order to provide suitable genetic material for assay of neoplastic potentialities. Tissue taken from an animal of an inbred strain should survive or grow in all animals of the same inbred strain (*33*). However, immunogenetic differences have been shown to develop between the cells in culture and animals of the inbred strain of origin (*2, 34*). Such differences can seriously impair the success of transplantation. Fortunately, however, the histocompatibility barriers are slight and can often be overridden by X irradiation of the host.

A second important factor in the successful transplantation of tissue-culture cells is the inoculum size. An excessively large inoculum is required for many long-term strains of tissue-culture cells, as compared with the numbers of cells needed for successful transfer of most tumors from animal to animal (*33*). There are probably several reasons for this requirement, especially in view of histocompatibility barriers and the different environmental conditions *in vitro* and *in vivo*. Probably the tumors develop from only a fraction of the implanted cells, either because the populations are heterogeneous and only a portion of the cells may survive and continue proliferation in the host or because a proportion of the cells during the period of adaptation will be destroyed by immune mechanisms in spite of their potentialities for further growth. In either case, increasing the inoculum size will favor early establishment of the graft.

From the results of the present study, a third factor that significantly influences the success of transplantation is the type of culture medium to which the cells are adapted. This finding appears to be true not only for these cell strains but also for other strains of cells, such as a strain of liver cells (*32*) and strain L (NCTC 2071). In previous assays of strain L (*18, 34*) the cells showed a progressive decline in their sarcoma-producing capacity during the years of culture in stock medium, to the point that most cultures failed to grow as sarcomas. However, recent assays of

comparable numbers of strain L cells adapted to protein-free, chemically defined medium NCTC 109 (*8*, *9*) have shown sarcoma production in untreated hosts from 50 percent of the cultures injected (5 out of 10).

The question immediately arises as to the nature of the change induced in these cell lines by the culture medium. Have the cells changed in their transplantability or in their neoplastic potentialities? Has growth in the new medium merely enhanced the resistance of the cells for transfer and survival *in vivo* or has culture in this medium increased the malignancy or autonomy of the cells? The observations seem to indicate an altered transplantability. Al[1] the observed changes in the cell lines induced by the different culture medium can be explained on the basis of a larger proportion of the injected cells surviving in the host—surviving both the host defenses and the change in nutritional environment. The adaptation to this medium, whether brought about through cell selection or induction, apparently better fitted the cells for single-cell growth *in vitro* and also for survival and continued growth when implanted in mice. After adaptation, the cell populations exhibited some shifts in chromosome-number distribution pattern (*5*). However, the cells still provided a competent antigenic stimulus to the host, were susceptible to the immunity induced, and maintained their distinctive morphology and glycolytic capacities *in vitro* (*6*). As compared with the high line, the low-line cells still grew slowly into palpable tumors and failed to elicit the early vascular reaction in the host that is associated with rapidly proliferating cell implants (*35*, *36*). This latter observation suggests that the new medium failed to alter significantly the proliferative capacity of the cells when first implanted in mice.

The enhanced tumor-producing capacity of the cells induced by the change in culture medium resembles, in certain respects, the changes in virulence [9] produced in microorganisms by growth in certain media (*37*). The virulence of microorganisms, like demonstrable malignancy of tissue-culture cells, depends on many different attributes of organism and host, any one of which can vary independently with resulting loss in virulence. When grown in different media, nutritional habits or antigenicity may change; also the physiologic state of the culture at the time of injection can affect its survival and adaptation to multiplication *in vivo*. For these reasons, it is not surprising that the maintenance of a culture of microorganisms in a virulent state outside the body is difficult. Similarly, the maintenance of a strain of tissue-culture cells in a demonstrably malignant state is also difficult, as shown with numerous long-term cell strains (*2*, *4*, *18*, *32*, *38*).

In these studies five clones were isolated from the high and low sarcoma-producing lines, which in turn arose from a single cell from normal tissue. All the clones produced sarcomas, indicating the carcinogenic change, which developed early during culture, became fixed in the cell population, and appeared to characterize the individual cells of each line. What

[9] The term "virulence" refers to the capacity of a microorganism to withstand defense mechanisms of the host, to multiply, and to produce a pathologic disturbance in the host.

induced this change in capacity of the cells to grow as autonomous neoplasms when implanted *in vivo*? Although the answer is unknown at the present time, several hypotheses have been suggested (*16*, *17*, *23*, *30*, *39–43*). A few of these will be considered.

Removal of cells from the physiologic controls regulating life in the multicellular organism and the use of culture media stimulating maximal proliferative capacity have presumably produced a drastic change in cell metabolism. In the differentiated organism, some of the proliferative capacity of many cell types appears to persist but to be kept in check by controls possibly hormonal or nutritional in nature. Removal of cells from such controls releases greater proliferative activity. Growth in a medium that cannot possibly contain all factors present in the tissue fluids must induce metabolic changes in the cells. Specific substrates can influence enzyme formation and activity and mediate synthetic changes, thus acting as differentiating agents. Such a permanent change in metabolic pattern may occur, just as in embryonic differentiation cells acquire relatively permanent and irreversible differences in morphology and function. From the population of metabolically altered cells, those best fitted for continuous growth *in vitro* or those that grow fastest would be selectively propagated. These cells may also exhibit autonomous growth if implanted *in vivo*.

A second possibility to account for the changed capacity of the cells to grow as autonomous neoplasms *in vivo* is that the rapid rate of cell proliferation *in vitro* favors the probability of gene mutations, redistribution of mitochondria, or the gradual dilution of cytoplasmic determinants, if such exist.

A third hypothesis is that the numerical and structural changes in chromosomes which occur in culture may cause the neoplastic change in certain cells, and these cells may be selectively favored during growth *in vitro* (*30*). The explantation of tissue cells from their normal position in the body into culture *in vitro* appears to result in mitotic disturbances with frequent breakage of chromosomes, deletions, and rearrangements. A genotypically diverse cell population results, with numerical and structural rearrangements of chromosomes. From this genetically heterogeneous population, the cells best adapted to continuous life *in vitro* survive, and these cells may also have the capacity for autonomous growth if implanted *in vivo*.

A fourth hypothesis is that some unidentified carcinogenic agent or agency, such as a chemical carcinogen, virus, or altered O_2 or CO_2 tension (*23*, *43*), may induce the carcinogenic change. With recent developments in chemically defined media and better controlled culture conditions, this hypothesis is most fruitful for further experimentation.

The present study has shown that individual cells from the high and low lines could reproduce in the derived clones some of the wide variations in chromosome numbers and types (*5*) and also many of the distinctive properties of their respective parent lines. Since the properties of the clones, in general, resembled those of the parent lines, it is con-

cluded from this analysis that the two lines do not differ merely in proportion of cell types present. Apparently cells of the two lines have different, relatively stable, heritable properties. In the maintenance of these properties, the exact complement of chromosomes appears unnecessary since a wide scatter of chromosome numbers in the hyperdiploid range characterized each line and clone.

How can we account for the different behavior of the two lines and their derived clones? Although cells of the two lines proliferated at approximately the same rate under culture conditions *in vitro*, the cells of the low line were not equally well adapted for continued proliferation on implantation into mice and they also exhibited a lower rate of anaerobic glycolysis *in vitro* (*6*, *44*). Recent studies by Scott and associates (*7*) showed that the high-line clone as compared with the low-line clones had consistently higher activities of three enzymes of the glucose-shunt mechanism, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and hexokinase. These observations suggest that the different metabolic and physiologic behavior of these clones may be associated with heritable differences in enzyme activity.

From the present study and those by Chu and associates (*5*) and Woods and associates (*6*), some differences also appeared between the low-line clones. One low-line clone (2555) that arose from a binucleate cell, the nuclei of which appeared to fuse, developed into a hypertriploid mononuclear cell population. Cells of this clone failed to grow on transplantation to nonirradiated hosts, but did grow as sarcomas in a few irradiated hosts. Whether this failure to grow in nonirradiated hosts resulted from heightened isoantigenicity, increased susceptibility to host immune factors, or a nutritional inadequacy in the host environment cannot be elucidated from data presently available. Another low-line clone (2470) exhibited a higher rate of anaerobic glycolysis, intermediate between that of high- and low-line cells, and thus appeared to be an intermediate in the progression toward an increased glycolytic capacity *in vitro* (*6*). At first, cells of this clone when implanted in mice also exhibited a shorter latent period for tumor development than cells of low-line clone 2445; recent tests showed a more prolonged latent period, which may be related to changes in cell transplantability. This clone also contained a large proportion of higher polyploid cells (37% of the cells higher than tetraploid), also characteristic of the high line and all high-line derivatives (*5*). These observations suggest a possible relationship, either direct or indirect, between the high rate of anaerobic glycolysis *in vitro* and the presence of higher polyploids in the cell population. The more limited studies on the fourth low-line clone (2426), isolated after mouse passage, failed to reveal any properties different from those of low-line clone 2445 that had not been grown *in vivo*. The influence of mouse passage on other characteristics of these cells has been discussed in another paper (*4*).

The advantage of the single-cell procedure used here over the easier plating technique used by Moen (*45*) and Puck and associates (*46*) is that

the isolation of one cell in a separate flask eliminates the possibility of contamination of the clone from cells of other colonies, either by migration or reanchoring of cells loosened into the supernatant fluid. This technique was introduced in 1948 before the development of improved culture media. The lack of an adequate medium at that time apparently accounted for much of the early difficulties in its use (*47*). With present culture media and with the minor facilitating changes in method noted, the procedure has proved highly reliable and easily used for many cell types of human, mouse, or chicken origin. A number of other single-cell procedures have since been developed (*48–51*).

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PLATES

PLATE 97

FIGURE 1.—Low-line clone (2445) photographed July, 1956, 35 days after isolation of single cell. *Observe* cells emerging from open end of capillary onto floor of Carrel flask. × 200

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PLATE 98

Representative areas of cultures of high and low lines. × 200

FIGURE 2.—Culture of high line in stock medium; 260th transplant generation, photographed December, 1957, after 7 years *in vitro*. *Note* elongated spindle-shaped cells and resemblance to morphology described previously (*1*).

FIGURE 3.—Culture of low line in stock medium; 286th transplant generation, photographed December, 1957, after 7 years *in vitro*. *Observe* general similarity to morphology described previously (*1*).

FIGURE 4.—Culture of low-line clone (2426) after mouse passage; 47th transplant generation *in vitro*, photographed June, 1957, 427 days after single-cell isolation. *Note* similarity to other low-line cells (fig. 3). *Observe* two large giant cells at *lower right* and *left*.

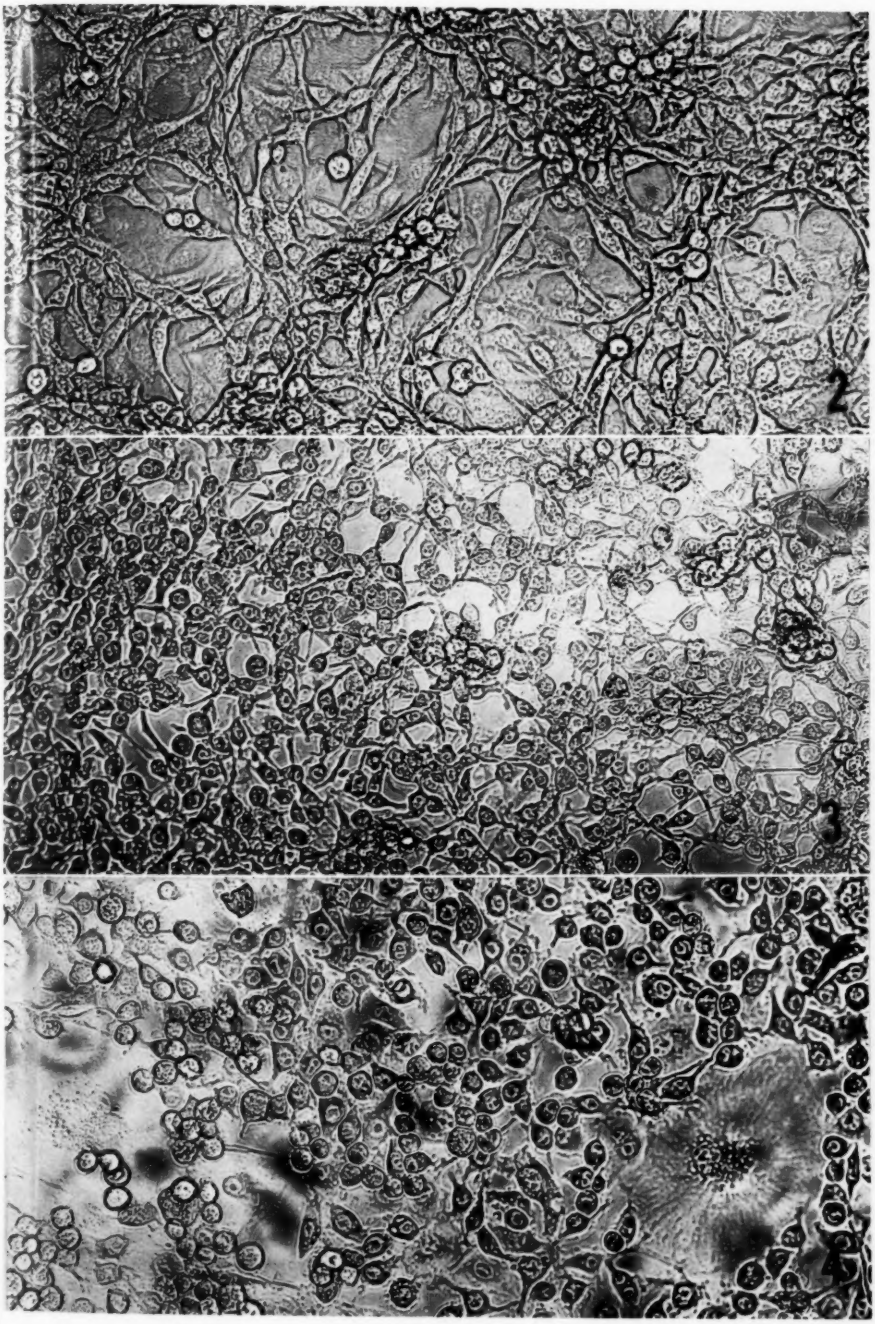
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PLATE 99

Representative areas of cultures of clones from high and low lines. × 200

FIGURE 5.—High-line clone (2472); 40th transplant generation, photographed January, 1958, 597 days after single-cell isolation. *Note* similarity in morphology to original line.

FIGURE 6.—Low-line clone (2470); 31st transplant generation, photographed November, 1957, 538 days after single-cell isolation. *Note* distinctive morphology as compared with figure 5.

FIGURE 7.—Low-line clone (2555); 21st transplant generation, photographed January, 1958, 479 days after single-cell isolation.

FIGURE 8.—Low-line clone (2445); 48th transplant generation, photographed November, 1957, 538 days after single-cell isolation.

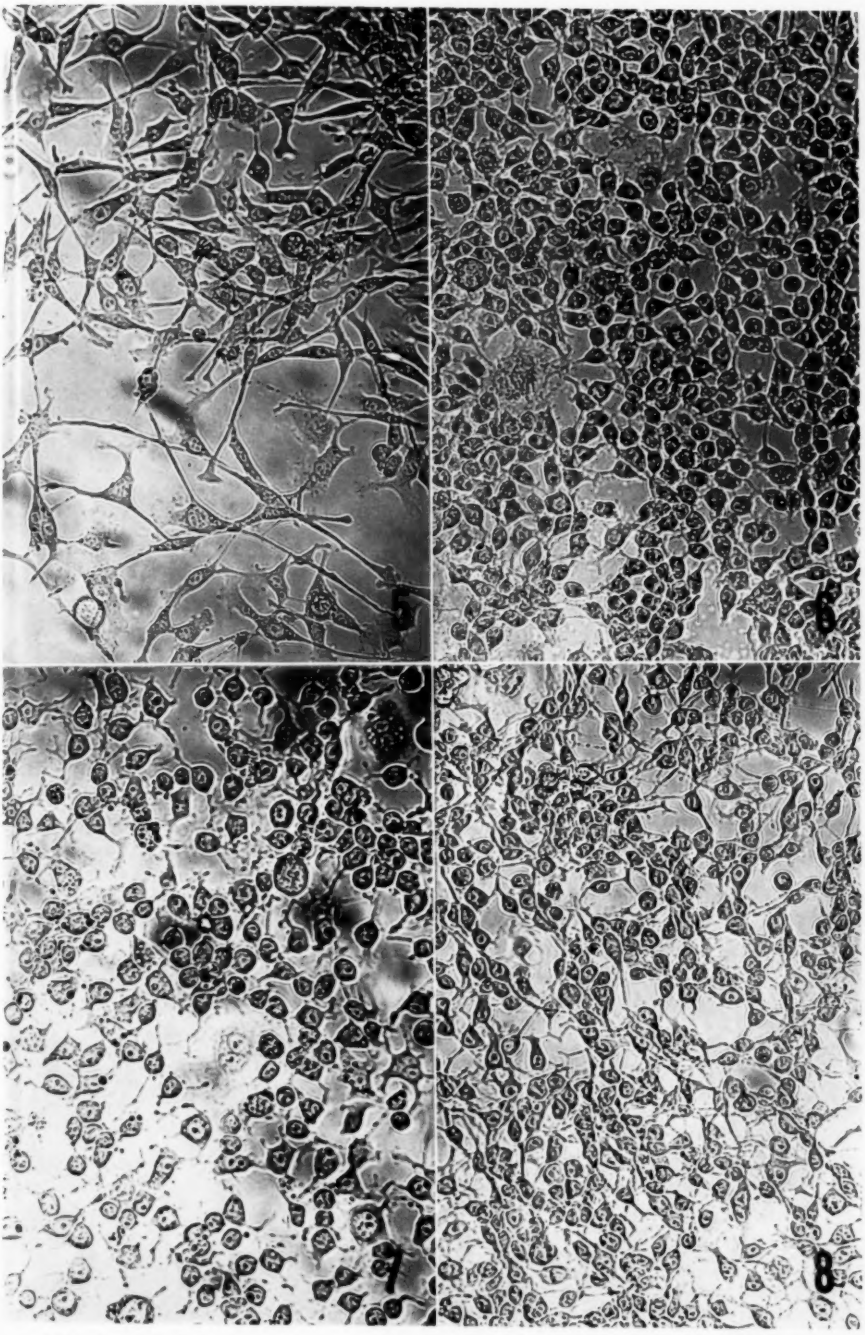
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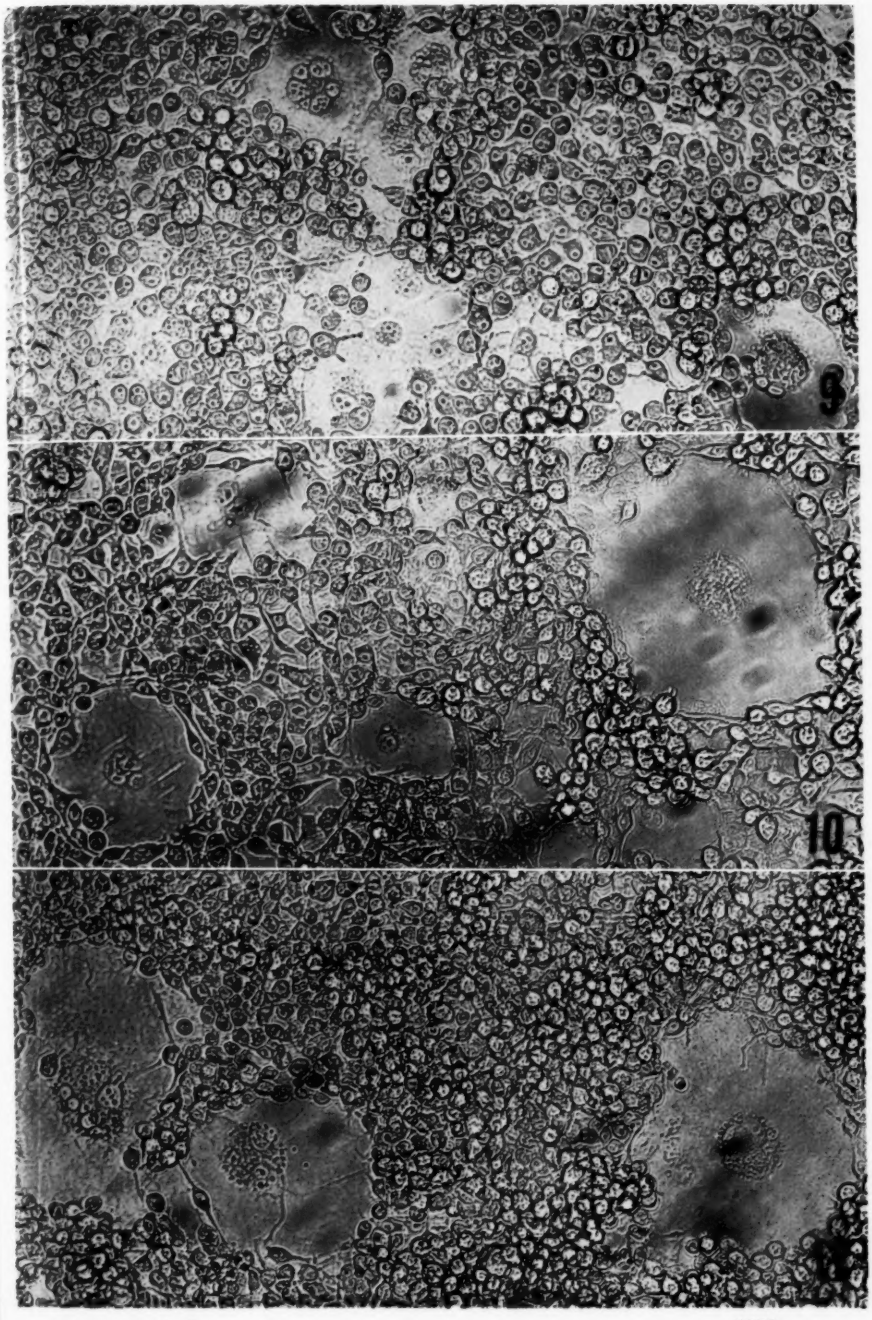
PLATE 100

Representative areas of low-line clones showing large giant cells. × 200

FIGURE 9.—Clone (2555); 21st transplant generation, photographed January, 1958, 479 days after single-cell isolation.

FIGURE 10.—Clone (2470); 53d transplant generation, photographed January, 1958, 597 days after single-cell isolation.

FIGURE 11.—Clone (2445); 41st transplant generation, photographed January, 1958, 597 days after single-cell isolation.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Influence of Animal Passage on a Line of Tissue-Culture Cells [1]

KATHERINE K. SANFORD, RUTH M. MERWIN, GWENDOLYN L. HOBBS, *and* WILTON R. EARLE,
Laboratory of Biology, National Cancer Institute,[2]
Bethesda, Maryland

SUMMARY

This study confirms and further elucidates a previous observation that mouse passage increases the tumor-producing capacity of a low tumor-producing line of mouse tissue-culture cells. The change in the cells was maintained during serial passage of the cells *in vivo* and persisted for several months after return to culture. The increased tumor-producing capacity ultimately subsided, partially in one strain and almost completely in another after 8 months *in vitro*. The cells before and after mouse passage did not differ significantly with respect to their morphology *in vitro* or *in vivo*, their failure to elicit an early vascular response in the host, their antigenicity for mice of the strain of origin (C3H), or their low rate of anaerobic glycolysis *in vitro*. Chromosomal numbers were slightly altered. The cells after mouse passage did seem to differ in their increased resistance to the immunity they induced in the host. This effect was produced not only in cells cultured on stock medium but also in cells cultured on a different medium known to enhance the transplantability of the cells. Two cell strains were isolated after mouse passage that exhibited the morphologic, metabolic, and physiologic characteristics of another line of cells carried independently from the low tumor-producing line but originated *in vitro* from the same cell. The observations suggest that this cell type was present in culture before mouse passage and was selectively concentrated during mouse passage. Several possibilities to explain the increased tumor-producing capacity of the cell population after mouse passage are discussed.—J. Nat. Cancer Inst. 23: 1061–1077, 1959.

PREVIOUS PUBLICATIONS (*1–3*) describe variant cell lines that arose *in vitro* from one cell that was cultured from normal subcutaneous, connective, and adipose tissue of an adult mouse. Cells of some of these lines, when implanted into mice of the strain of origin (C3H), grew as sarcomas in almost every host, whereas cells of other lines grew as tumors in only a few or in none of the mice injected. Cells of the low sarcoma-producing line or "low" line,[3] which produced tumors in a few of the

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[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

[3] This line was originally referred to as a culture line of substrain III (*1*). However, as carried in our laboratory, this line is designated NCTC strain 2049.

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mice, produced a larger number of tumors when injected into irradiated hosts. The resulting tumors grew progressively when inoculated into nonirradiated strain C3H mice. Thus, after one mouse passage, the transplantability or malignancy of low-line cells for C3H mice was significantly increased.

The following questions have been considered in the present study:

(1) Would the change in the cell population observed after one mouse passage persist *in vitro* after returning the cells from mice to culture?

(2) Did low-line cells after mouse passage differ in any other properties from low-line cells not grown in mice? Distinctive properties of low-line cells were described previously (*1*, *2*), and included a characteristic morphology *in vitro* and *in vivo*, a prolonged latent period for tumor development *in vivo*, antigenicity for strain C3H mice, susceptibility to the immunity induced, failure to elicit an early vascular response in the host, a low rate of anaerobic glycolysis *in vitro* (*4*, *5*), and a characteristic chromosomal constitution (*6*). The glycolytic properties and chromosomal constitution of the cells were investigated by Woods and associates and Chu and associates, respectively, and will be only briefly summarized here.

(3) A previous study (*7*) showed that prolonged culture of low-line cells in serum-supplemented medium NCTC 109 enhanced the transplantability of the cells to strain C3H mice. Would mouse passage further enhance the transplantability of these cells?

(4) What is the nature of the change increasing the tumor-producing capacity of the cell population during mouse passage? Possible answers to this question have been considered in the discussion.

MATERIALS AND METHODS

The general procedures for cell culture and for assay of the cells in X-irradiated or nonirradiated mice have been described (*2*). Cells were grown in T-60 flasks (*8*) under perforated cellophane or on the glass floor of the flask in a culture fluid, designated "stock medium," consisting of 40 percent horse serum, 20 percent chick-embryo extract (1:1.25), and 40 percent Earle's saline. In one experiment, a clone from the low line [NCTC clone 2445 (*7*)] was used, and this clone was cultured in chemically defined medium NCTC 109 (*9*, *10*), supplemented with 10 percent horse serum. This medium will be referred to as "experimental medium."

To assay tumor-producing capacity, the cells were suspended in culture fluid and injected in 1-ml. aliquots into thigh muscles of strain C3H/HeN mice. In the early assays, the cells from one T-60 flask culture were injected into one or more mice. From determinations of the average number of cells per dense culture, the size of inoculum could be estimated. In all later assays, the size of inoculum was determined by enumerating cell nuclei in aliquots of the individual suspensions injected (*11*). After injection, the mice were observed for 3 to 4 months. To determine the

tumor latent period, each mouse was examined weekly for any palpable growth at the injection site. The tumor latent period measured the interval from injection of cells to the appearance at the injection site of a palpable tumor that continued to grow as a diagnosed sarcoma. Diagnosis of sarcoma was made by microscopic examination of tumor sections stained with hematoxylin and eosin and with van Gieson's picrofuchsin.

The growth and vascularization of cell implants were studied in living mice by means of a transparent-chamber technique developed by Algire and Legallais (*12*) and modified by Merwin to facilitate observation of tissue-culture cells (*2*).

EXPERIMENTS AND RESULTS

Permanence of the Change Produced by Mouse Passage

The object of the first experiments was to determine whether the increased tumor-producing capacity of the cell population after mouse passage persisted during subsequent growth of the cells *in vitro*. Strains of cells were cultured from 11 different sarcomas. These sarcomas arose from injections of low-line cells into strain C3H/HeN or F_1 hybrid (BALB/c $\times$ C3H/HeN) mice,[4] most of which were irradiated.

Although it was recognized that a single F_1 passage might modify the immunological characteristics of the cells (*13–15*), the hybrid mice were used in some experiments to determine whether tumors produced by cells after mouse passage originated from low-line cells or from the host stroma. Cells from the hybrid host would not, as a rule, be transplantable to C3H mice (*16*); therefore, tumors developing in C3H hosts from cells after hybrid passage would presumably have originated from low-line cells. Evidence from these experiments indicated that the altered cells originated from the low line and not from the host stroma, and there appeared to be no striking difference in behavior between cells passed through the hybrid as compared with those passed in C3H mice.

After periods of culture ranging from 11 to 140 days (1 to 14 transplant generations), cells of the 11 sarcoma strains, as well as control low-line cells that had not been grown in mice, were assayed in strain C3H mice. The results of these assays are summarized in table 1.

The results showed clearly that cells cultured for periods up to 4½ months after mouse passage produced a higher incidence of tumors, usually with somewhat reduced latent periods, than did comparable numbers of low-line cells that had not been grown in mice.

Two of the cell strains, 1935 and 2432, were continued in culture for almost a year. Results of assaying these cell strains after 240 to 330 days (21 to 29 transplant generations) are compared in text-figure 1 with results of assaying low-line cells before mouse passage. Cells of strain

[4] The authors express their appreciation to Dr. Clifford Grobstein, formerly of the Laboratory of Biology of this Institute, now at Stanford University, who supplied the hybrid mice used. These mice were 1- to 2 month-old males.

TABLE 1.—Tumor production by low-line cells not grown in mice and by low-line cells cultured for 11 to 140 days after mouse passage

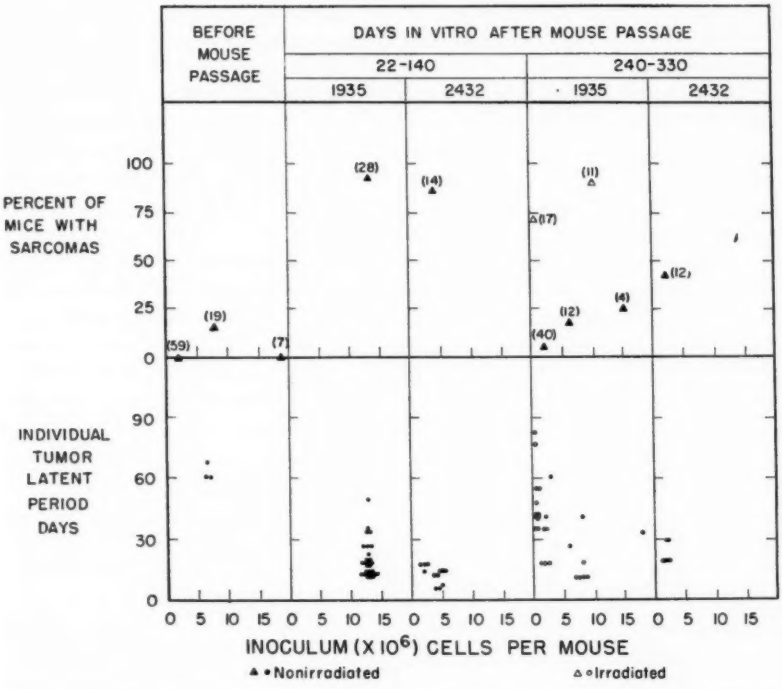
Cells assayed	Inoculum size ($\times 10^6$ *cells per mouse*)	Strain designation	Mice with tumors: Number of mice injected	Tumor latent period (*days*): Range	Average
Low line not grown in mice	2	2049	0/59	—	—
	4		1/10	55	55
	6		1/8	84	84
	7		0/8	—	—
	8		3/19	61–68	63
	11		1/12	63	63
	12		0/12	—	—
	16		1/12	42	42
	19		0/7	—	—
	Total.....		7/147 (5%)		
Low line after mouse passage	1	2185*	3/3	15–22	17
		2186*	3/3	15–49	38
		2314†	1/3	19	19
		2354‡	1/5	90	90
	2	2432	4/6	15–18	17
	3	2229‡	1/5	26	26
	4	2123‡	1/3	56	56
		2124‡	2/3	15–78	47
		2432	4/4	6–13	10
		2355‡	1/3	36	36
	5	2432	4/4	7–14	12
	10§	2123‡	2/2	12	12
		2124‡	1/2	54	54
	13§	1935†	26/28	13–50	20
	20§	1894	2/2	14	14
		2123‡	2/2	6	6
		2124‡	4/4	6–13	8
	Total.....		62/82 (76%)		

*Cell strain from tumors arising in nonirradiated mice.
†Cell strain from tumors carried for 2 to 4 generations *in vivo*. All other strains were from first-passage tumors.
‡Cell strain from tumors in F_1 hybrids (BALB/c × C3H).
§Inoculum size estimated. *See* "Materials and Methods."

1935 showed a decline in sarcoma-producing capacity almost to a level characteristic of the original low line. Cells of strain 2432 showed less of a decline in tumor production, and the latent periods were shorter. This strain was also found to differ in certain other characteristics to be noted later.

A sarcoma from low-line cells was carried for over a year through 8 passages in nonirradiated strain C3H mice. Of 38 mice inoculated during this period, each developed a sarcoma at the site of inoculation, and tumor latent periods averaged 2 weeks.

In summary, these experiments showed that the increased tumor-producing capacity, to 100 percent takes, induced by mouse passage was maintained during serial passage of the cells *in vivo*. Some of this increase persisted for several months after return to conditions *in vitro*, but ulti-



TEXT-FIGURE 1.—Tumor production by low-line cells before mouse passage and by sarcoma strains 1935 and 2432. *Lower panel* shows individual latent periods for development of tumors plotted against inoculum size. *Top panel* shows percentage of mice developing sarcomas. For each inoculum-size group, the percentage of mice developing sarcomas was plotted against mean inoculum received by mice in the group. Number in parentheses indicates number of mice receiving injections. Strain 1935 cells were assayed in X-irradiated as well as nonirradiated mice of strain C3H, as indicated.

mately subsided partially in one strain and almost completely in another after 8 months of culture.

Characteristics of Cells After Mouse Passage

Further studies compared the morphology of cells *in vitro* and *in vivo*, before and after mouse passage, to determine whether any change in cell appearance or growth pattern occurred as a result of mouse passage. One of the 11 sarcoma strains listed in table 1 (strain 2432) and another strain not listed (strain 2632) differed from typical low-line cells, but the other 10 showed no detectable change (figs. 1 through 6).[5] The two strains showing the different morphology also differed in other characteristics to be noted later. Cells of these two strains resembled in

[5] The authors express their appreciation to Dr. Thelma Dunn of the Laboratory of Pathology of this Institute for her valuable assistance in comparing the tumor sections.

morphology cells of another line originated from the same cell as the low line and described previously as the high sarcoma-producing line or "high" line [(*1*, *2*); figs. 7 and 8]. Certain cultures of the low line, particularly in March and April, 1956, were observed to contain cells resembling those of the high line (figs. 9 and 10) and to produce tumors like those from high-line cells. From two such tumors, strains 2432 and 2632 were isolated. Strain 2432 was noted earlier as a strain of cells producing a high incidence of sarcomas with shorter latent periods (text-fig. 1).

A group of experiments tested whether the increased tumor-producing capacity of low-line cells after mouse passage resulted from the cells having lost their antigenicity for strain C3H mice. The following cells were tested for their capacity to immunize strain C3H mice against a second injection of the cell types indicated in table 2: (1) cultures of low-line cells not grown in mice, (2) tumor tissue from mice injected with low-line cells, and (3) cells cultured from a low-line tumor.

TABLE 2.—Tests for capacity of low-line cells before, during, and after mouse passage to immunize strain C3H mice against a second injection of cells

Cells used for first injection	Results of challenge: Mice with sarcomas / Number of mice challenged					
	Cells used for challenge					
	Low line before mouse passage		Low-line tumor		Strain 1935 from low-line tumor	
	Injected	Non-injected	Injected	Non-injected	Injected	Non-injected
Low line before mouse passage	0/17*	11/20	6/6	6/6	—	—
Low-line tumor	0/5	6/6	6/6†	6/6	—	—
	0/8	5/8	6/6†	6/6	—	—
	0/6	6/6	6/6	5/5	—	—
	—	—	6/6	6/6	—	—
Strain 1935 from low-line tumor	0/5	4/5	—	—	2/3	1/4
	—	—	—	—	3/6‡	4/7
	0/4	2/4	—	—	2/12§	4/4§

*In one mouse a sarcoma developed at site of immunization.
†First injections were made intramuscularly into thigh or foreleg rather than tail.
‡First injections were made subcutaneously into foreleg rather than tail.
§Experimental and control mice were X irradiated before challenge.

For the first injection, the following methods of inoculation were used for the three types of cells:

(1) Low-line cells from culture were injected into the foreleg muscles as a suspension in 1 ml. of culture fluid.

(2) Tumor tissue from mice was inoculated intramuscularly into thigh or foreleg or subcutaneously into the tip of the tail. The tumors had been carried for 3 to 6 serial passages in nonirradiated strain C3H mice. Growths resulting from the tail inoculations were small or not visible grossly.

(3) Cells cultured from a tumor, strain 1935, were injected subcutaneously into the tail as a concentrated suspension in 0.1 ml. of culture fluid, or subcutaneously into the foreleg as a suspension of 10^6 cells in 1 ml. of culture fluid. No tumors developed. The cells had been carried for 5 to 9 months *in vitro*.

Two weeks after the first injection, both the injected mice and non-injected control mice of the same age were challenged by implanting cells of various types (table 2) into the thigh muscles. The inoculum size for all cultured cells, either before or after mouse passage, was approximately 10^7 cells per mouse. Mice challenged with low-line cells were X irradiated with a whole-body dose of 425 r [6] before injection, since low-line cells generally fail to grow in nonirradiated hosts. In the last experiment (table 2), the same group of injected mice was challenged with either low-line cells or cells of strain 1935. Results of challenge are summarized in table 2.

A first injection of cells that had grown in mice (1) completely prevented tumors from a second injection of cells never grown in mice, (2) failed to prevent tumors from a second inoculation with tumor tissue, and (3) failed to prevent, completely, tumors from a second injection of "mouse-passaged" tissue-culture cells where cell dosage was approximately the same as that of cells never grown in mice. These results suggested that cells after mouse passage, while still providing a competent antigenic stimulus to the mouse, were less susceptible to the immunity induced.

Cells cultured from the sarcomas were implanted in transparent chambers in C3H mice to determine whether the cells after mouse passage still failed to initiate a vascular response in the host. A previous study (*2*) showed that implants of low-line cells never induced the enlargement and convolution of nearby host blood vessels regularly produced by implants of the high sarcoma-producing line. Of the 8 strains examined after mouse passage, only 2 strains (2432 and 2632) initiated a strong vascular reaction in the host similar to that observed with high-line cells. Cells of the other 6 strains either elicited no reaction at all or a very weak reaction near a localized area of the implant. These conclusions were based on an examination of 22 mice, 9 implanted with cells of strains 2432 and 2632, tested after 3 to 11 months of culture, and 13 implanted with cells of the other 6 strains, tested after 2 weeks to 13 months of culture.

A previous study by Woods and associates (*4*) had shown that cells of the low line had a low rate of anaerobic glycolysis *in vitro* about one third that of cells of the high sarcoma-producing line. Glycolytic rates of the cells after mouse passage did not differ significantly from those of the cells that had not been grown in mice except for strains 2432 and 2632, which exhibited glycolytic rates in the range characterizing cells of the high sarcoma-producing line (*5*).

The chromosomal constitution of two sarcomas developed from low-line cells injected into strain C3H mice, and examined by Chu and associates

[6] The authors express their appreciation to Mr. Henry Meyer of the Radiation Branch of this Institute, who irradiated the mice according to the procedures described previously (*3*).

(*6*), resembled the chromosomal constitution of the cells before mouse passage. Both populations of cells showed a wide range of chromosome numbers in the hyperdiploid range and characteristic structural alterations, such as metacentrics and chromosomes of abnormal size. The modal number of chromosomes was slightly higher in the sarcoma than in cells *in vitro*, but additional data would be necessary to establish this difference as a consistent result of mouse passage.

In summary, these studies suggested that low-line cells before and after mouse passage differed in susceptibility to the immunity they induced in the host. In addition, possibly a slight difference in chromosome numbers might be significant. Two of the 12 strains exhibited morphologic, metabolic, and physiologic characteristics of high-line cells. Because these 2 strains originated from tumors resembling those of the high line, and because the tumors were developed at a time when certain cultures *in vitro* exhibited high-line morphology, it seems probable that mouse passage merely concentrated cell types already present in culture. After mouse passage, cells of one of these strains (2432) showed some decline in sarcoma-producing capacity with time in culture (text-fig. 1). Similarly, cells of the high sarcoma-producing line also exhibited some decline in sarcoma-producing capacity with years in culture, as reported previously (*2*).

Effect of Mouse Passage on Cells Cultured in Experimental Medium

The next experiment tested whether mouse passage could further enhance the transplantability of cells cultured in experimental medium. A previous study (*7*) showed that an injection of cells that had been cultured in this medium, whether taken directly from culture or from growth in mice, induced immunity in C3H mice effective against a second injection of the same cell type not grown *in vivo*. Depending on cell dosage, this immunity either prevented growth of tumors or prolonged the tumor latent period.

To test for further enhancement of these cells by mouse passage, NCTC clone 2445 from the low line was used (*7*). Strain C3H mice were immunized by implanting a small piece of first-passage tumor in the tail. Two weeks later, both the immunized and nonimmunized controls were challenged by injecting intramuscularly either (1) clone cells not grown in mice or (2) clone cells cultured for 2½ weeks after mouse passage. The results of these experiments are summarized in table 3.

With the number of cells used for challenge, there was only a slight effect, if any, of immunization on incidence of tumors produced but a significant effect on tumor latent periods. The latent periods for tumors arising from cells not passed through mice were significantly prolonged by previous immunization, whereas no effect was observed on the latent periods for cells obtained after mouse passage. These results confirmed the earlier indication that cells after mouse passage are less susceptible to

TABLE 3.—Influence of mouse passage on transplantability of clone 2445 cells cultured in experimental medium

Cells used for immunization	Cells used for challenge									
	Clone not grown in mice					Clone after mouse passage				
	Inoculum ($\times 10^6$ *cells per mouse*)	Results of challenge				Inoculum ($\times 10^6$ *cells per mouse*)	Results of challenge			
		Mice with sarcomas / Number of mice challenged		Tumor latent period Average and range (*days*)			Mice with sarcomas / Number of mice challenged		Tumor latent period Average and range (*days*)	
		Immunized	Nonimmunized	Immunized	Nonimmunized		Immunized	Nonimmunized	Immunized	Nonimmunized
Clone 2445 tumor	13. 4	8/10	5/5	40 (22–46)	14 (11–26)	8. 2	9/10	6/6	11 (all 11)	11 (all 11)

acquired immunity, and extended the conclusion to cells cultured in experimental medium.

DISCUSSION

What is the nature of the change increasing the tumor-producing capacity of the cell population during mouse passage? Several possibilities will be considered. It seems clear that during mouse passage the cells acquire increased resistance to the immunity they induce in the host. This increased resistance could conceivably account for the enhanced tumor-producing capacity of the cell population. Increased resistance to acquired immunity might result from (1) immunoselection of more resistant cells already present in the tissue-culture cell population, (2) adaptation of the cells to growth in mice, or (3) "immunologic enhancement."

Variations between cells in culture, possibly at the cell surface, might differentiate immunologically sensitive from resistant cells, and the latter would presumably be concentrated by immunoselective pressures *in vivo*.

The second possibility is that, during growth in the mouse, the cells might acquire metabolic mechanisms better adapting them for survival and rapid proliferation in mouse tissues. Such an adaptation could result from selection of more competent nutritional or metabolic variants from a mixed-cell population; or substrates *in vivo* might induce adaptive changes in enzyme activities and metabolic pathways only gradually reversed during subsequent culture *in vitro*. As a result of this adaptation, a larger fraction of the cells inoculated would survive and proliferate. This would increase the effective inoculum size, which, in turn, would increase the success of transplantation to previously immunized hosts (*7*). It has long been recognized that highly malignant, rapidly growing tumor transplants can often override immune defenses of the host (*17*). However, even after mouse passage, these cells, as compared with those of the high sarcoma-producing line, still grew relatively slowly into palpable tumors and still failed to elicit the early vascular response in host vessels usually associated with implants of rapidly proliferating cells (*18*).

The third possibility is that "immunologic enhancement" occurred. The term "immunologic enhancement" was applied by Kaliss and Bryant (*19*) to the phenomenon of progressive growth of a homograft in mice actively immunized with tissue homogenates or passively immunized with antitissue serums. From their analysis of this phenomenon, contact of the graft with antiserum appeared to initiate a long-lasting alteration in the tumor cells that ensures their survival in the resistant host. Since low-line cells are immunologically incompatible with the host, this phenomenon might occur.

Further studies are in progress in an effort to establish which, if any, of these possibilities could be responsible for the change in the cell population.

Although resistance to immunity seems to increase during mouse passage, it is also possible that some loss in antigenicity may occur. The

cells could become less antigenic through the rapid dilution and loss *in vivo* of foreign proteins (horse and chicken) acquired *in vitro* from the heterologous culture fluid. However, it was shown in a previous study (*2*) that the immunity is induced specifically by the cells and not by the heterologous culture fluid. An alternative concept is that through immunoselection less antigenic cell types already in the mixed tissue-culture cell population could be selectively concentrated. Although some such decrease in antigenicity may have occurred, the cells, after six serial generations of growth in mice, were still sufficiently antigenic to immunize the host against a second injection of the same cell type not grown *in vivo*.

The increased tumor-producing capacity of the cell population, brought about by mouse passage and gradually reversed on subsequent culture of cells *in vitro*, appears not to be limited to this line of tissue-culture cells. A similar reversible change was reported for a clone of mouse liver cells that had undergone malignant transformation in culture (*20*). Such modifications in tumor-producing capacity obtained by shifting the cultural system from one *in vitro* to one *in vivo* and *vice versa* resemble in some respects the increases and attenuations in virulence [7] produced in microorganisms by animal passage and by propagation in certain culture media, respectively (*21*). Possibly the nature of some of these changes in microorganisms and in mammalian-tissue cells *in vitro* may be similar.

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PLATES

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PLATE 101

Morphologic comparison of tumors produced by low-line cells before and after mouse passage. The sections were stained with hematoxylin and eosin. × 335

FIGURE 1.—Representative area of sarcoma from which cells of strain 1894 were cultured.

FIGURE 2.—Representative area of sarcoma developed from injecting cells of strain 1894 into a strain C3H mouse after cells had been cultured for 21 days *in vitro*. *Note* morphologic similarity to tumor of figure 1.

FIGURE 3.—Representative area of sarcoma from which cells of strain 1935 were cultured.

FIGURE 4.—Representative area of sarcoma developed from injecting cells of strain 1935 into a strain C3H mouse after cells had been cultured for 22 days *in vitro*. *Note* general morphologic similarity to tumor of figure 3.

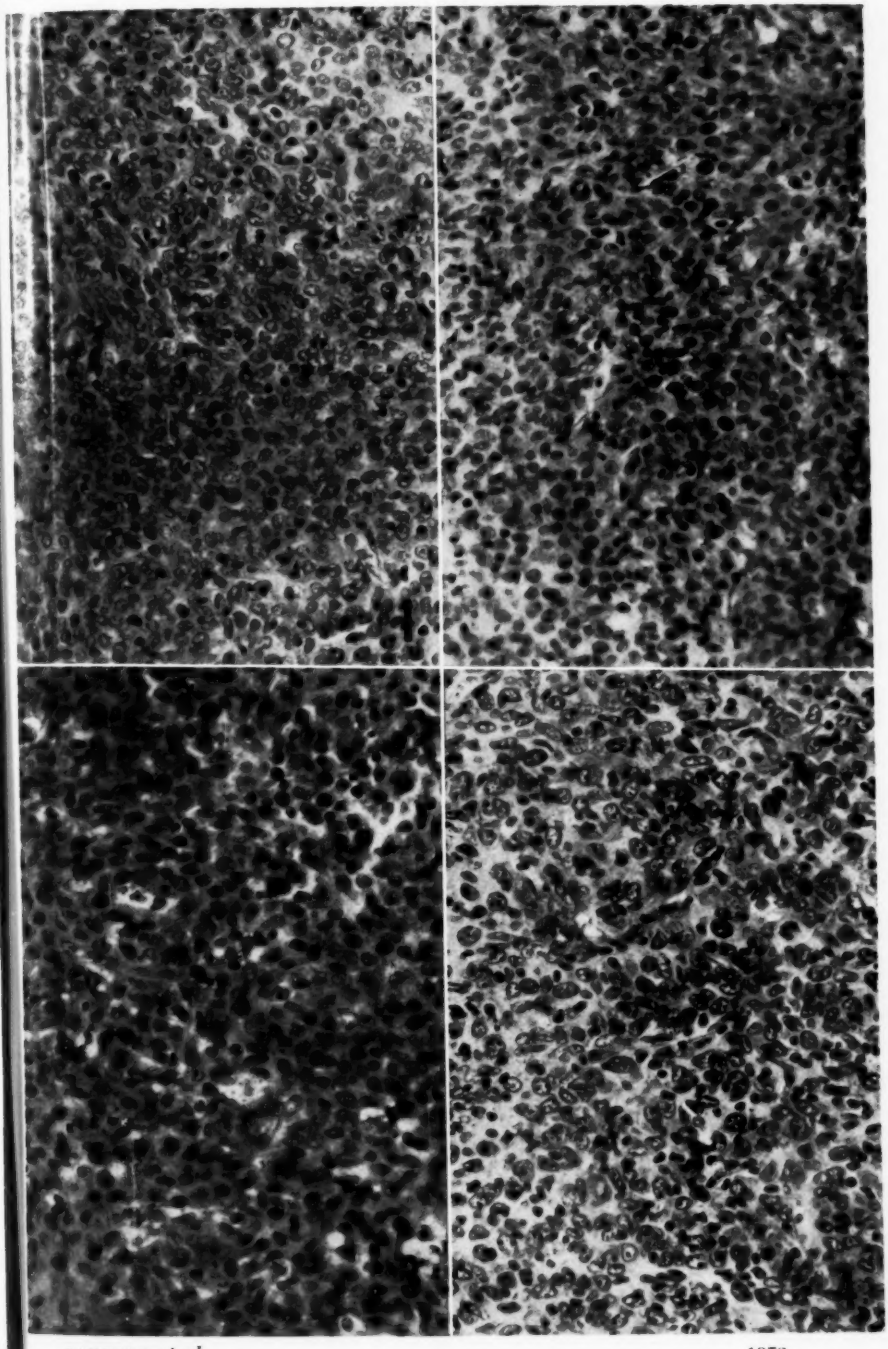

PLATE 102

FIGURE 5.—Representative area of culture of strain 2355 photographed 13 days after planting. This culture was in second transplant generation and cells had been cultured *in vitro* for 33 days. *Note* characteristic morphology of low-line cells. × 200

FIGURE 6.—Representative area of sarcoma developed from cells of strain 2355 in irradiated mouse of strain C3H. Cells used for injection were from a single cell of strain 2355 [NCTC clone 2426 (7)] and were in the fourth transplant generation. *Note* round nuclei with prominent nucleoli characteristic of tumor cells of the low line (*1*). These tumors were hemorrhagic and soft. Hematoxylin and eosin. × 335

FIGURE 7.—Cells of strain 2432 photographed after 2 months *in vitro* and 2 transplant generations. *Note* more elongated spindle-shaped cells as compared with those of figure 5. × 200

FIGURE 8.—Representative area of sarcoma developed from cells of strain 2432. *Note* more oval and fusiform cells arranged in bundles, the morphologic pattern resembling that of tumors from high-line cells (*1*). These tumors were more solid and firm in consistency. Hematoxylin and eosin. × 335

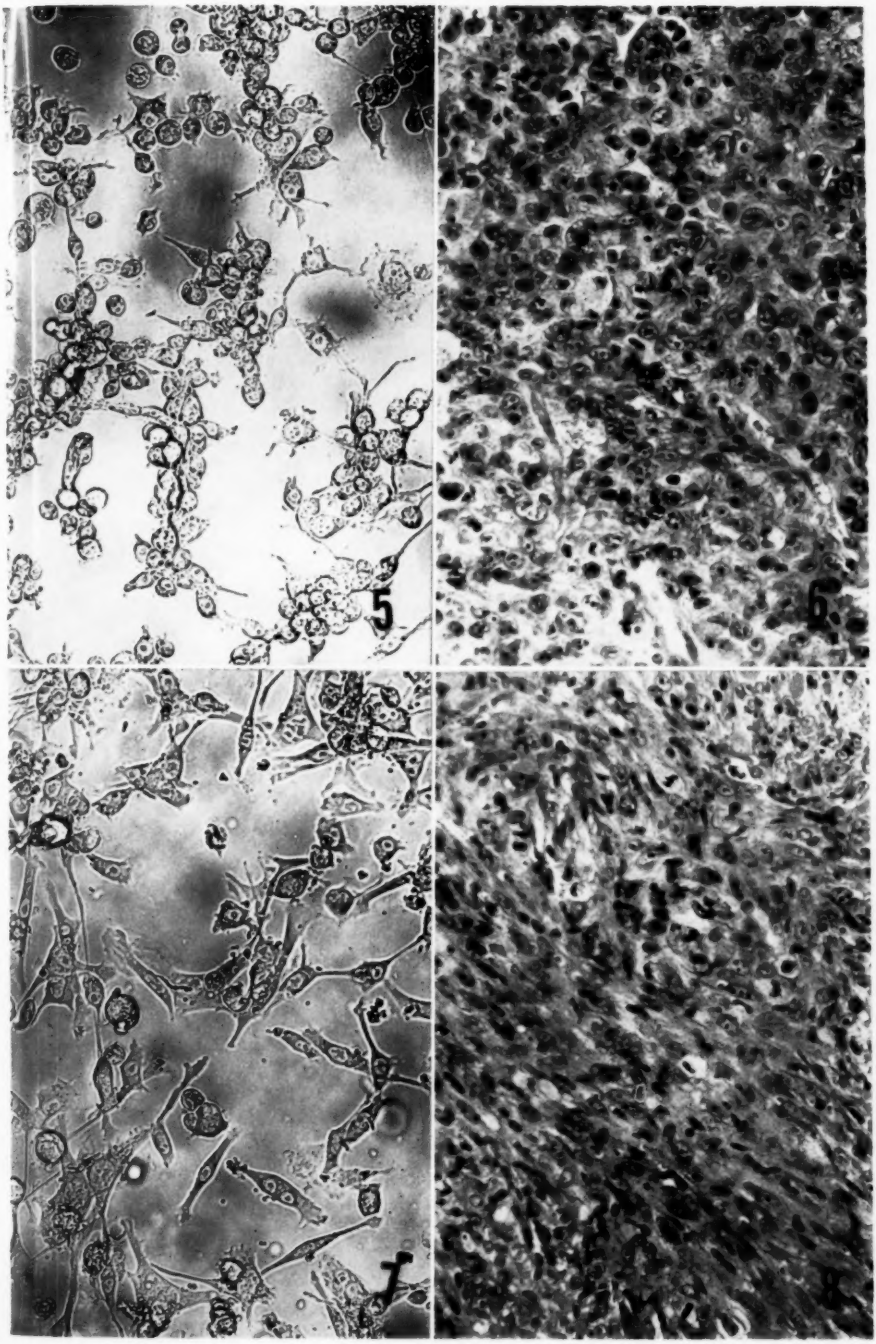
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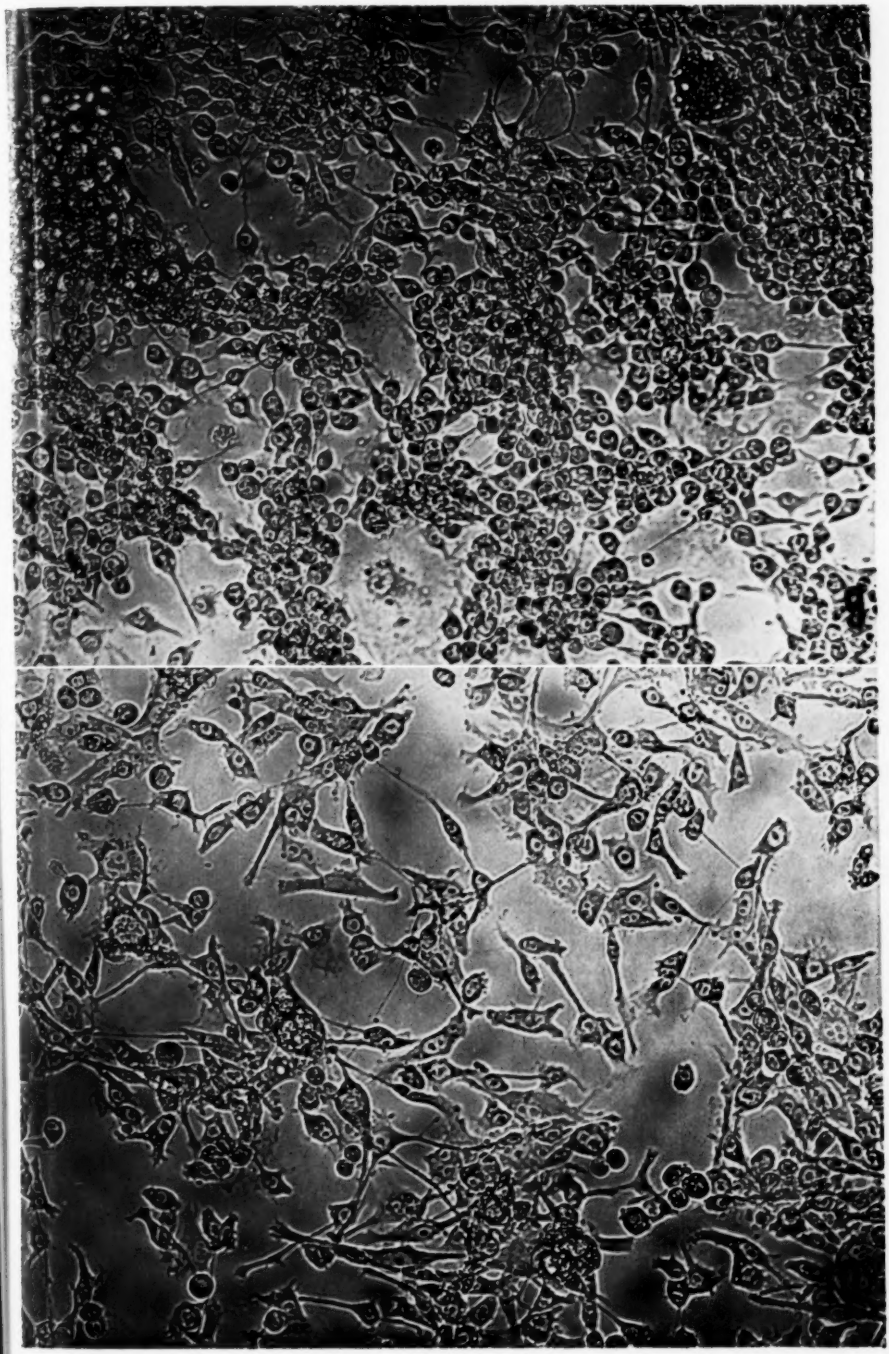
PLATE 103

FIGURE 9.—Representative area of a culture of low-line cells photographed 15 days after planting and approximately 5 years after single-cell isolation. *Note* characteristic morphology described earlier (*1*) and presence of large giant cell at *bottom* of figure. × 200

FIGURE 10.—Representative area of a culture of low-line cells photographed 6 days after planting, showing more elongated spindle-shaped cells observed occasionally in certain low-line cultures. This culture was photographed approximately 5 years after single-cell isolation. × 200



Glycolytic Properties of High and Low Sarcoma-Producing Lines and Clones of Mouse Tissue-Culture Cells [1]

MARK W. WOODS,[2] KATHERINE K. SANFORD,[2] DEAN BURK,[2] *and* WILTON R. EARLE,[2] *Cytochemistry Section, Laboratory of Biochemistry, National Cancer Institute,*[4] *and Tissue Culture Section, Laboratory of Biology, National Cancer Institute,*[4] *Bethesda, Maryland*

SUMMARY

Comparative anaerobic-glycolysis measurements were made of mouse sarcoma-producing cell lines, their clonal derivatives, and strains cultured from sarcomas produced by injection of these lines into mice of the original strain. These various sublines were all derived originally from one and the same cell cultured from normal subcutaneous connective adipose tissue. Sarcoma-producing cell lines of low tumor-producing potency had low glycolytic quotients (avg. $Q_{CO_2}^{N_2} = 36$–42), whereas lines of high sarcoma-producing capacity had high glycolytic quotients (avg. $Q_{CO_2}^{N_2} = 93$–110). One clone of intermediate glycolytic activity (avg. $Q_{CO_2}^{N_2} = 58$) displayed variable tumor latent periods. Tests were also made on two strains of fibroblasts recently isolated from subcutaneous adipose tissue of C3H mice. These recently isolated fibroblasts lacked the ability to produce tumors on reinjection into mice, and had relatively low glycolytic activities (avg. $Q_{CO_2}^{N_2} = 33$). Decreased sensitivity of glycolysis *in vitro* to inhibition by a specific anti-insulin (*i.e.*, anti-hexokinase) inhibitor, a *Podophyllum* derivative, was associated with increased glycolytic and sarcoma-producing capacities. The possible role of the mitochondria as the loci, and hereditary carriers, of the glycolytic and other possible changes is considered.—J. Nat. Cancer Inst. 23: 1079–1088, 1959.

SARCOMA-PRODUCING cell lines were derived *in vitro* from a single mouse cell of normal tissue origin (*1–3*). These cell lines differed in tumor-producing capacity when injected into the mouse strain of origin. The present paper reports comparative determinations of the glycolytic characteristics of some of these sarcoma-producing cell lines before and after mouse passage. Preliminary reports of some of the findings have already appeared (*3*, addendum to *4*). The data further support the thesis, first presented by Warburg (*4, 5*), that progression toward malignancy is associated with an increased rate of glucose metabolism.

Previous work with certain mouse tumors (*6–9*) has shown that the

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[2] Cytochemistry Section.

[3] Tissue Culture Section.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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extent to which the host can regulate tumor growth, *e.g.*, during temperature stress, is dependent upon the degree of sensitivity of the tumor cells to regulation by the insulin:anti-insulin hormonal system. Thus, when mice bearing the Cloudman S91 melanoma are exposed to a stressful temperature (35–40° C.), tumor glycolysis measured *in vitro* is rapidly lowered some 50 percent, and tumor growth in the mouse stops. The glycolytic rate can be restored *in vitro* to the control level by addition of insulin, providing the stress was not excessive. These effects are not due to a direct action of temperature on the tumor *per se*, but rather result from the intensification of host hormonal mechanisms which depress the rate of glucose phosphorylation in an insulin-reversible manner (*7*, *8*). Hochstein (*10*, *11*), working with subcellular preparations of mouse melanoma, has shown that this stress-modifiable insulin:anti-insulin regulation of glycolysis takes place at the level of the mitochondrial hexokinase. He was able to show that insulin and such anti-insulins as testosterone or *Podophyllum* fraction CC7E exert their characteristic effects in glycolyzing mitochondrial preparations, but not in solubilized glycolytic systems. Insulin : anti-insulin regulation of glycolysis in mitochondrial preparations was by-passed by adding glucose-6-PO_4, the product of the hexokinase reaction.

Certain *Podophyllum* derivatives have a steroid-like inhibiting action on glycolysis (*7*, *9*, *12*, *13*). The *in vitro* sensitivity of tumor-cell glycolysis to these agents, or to certain steroids, is correlated with the sensitivity of the tumor to regulation of its glycolysis and growth *in vivo* by the host (*6–8*). Thus it was found that a series of tumors which formed a spectrum of sensitivities to glycolytic inhibition by *Podophyllum* fraction *in vitro* also formed a parallel spectrum with respect to their abilities to grow in temperature-stressed mice. In the present study of tissue-cell lines maintained *in vitro*, it was found that the increased glycolysis which accompanies accentuation of tumor-producing properties was associated with a lessened sensitivity of the glucose metabolism to an anti-insulin inhibitor of the type just described.

MATERIALS AND METHODS

The origin and characteristics of the tissue-culture lines and clones used in this study have been described (*1–3*, *14*, *15*). The high and low sarcoma-producing lines, designated NCTC strains 1742 and 2049, respectively, were two lines of cells derived from one cell cultured from normal subcutaneous adipose tissue of an adult strain C3H/He mouse. The terms "high" and "low" refer to the relative incidence of sarcomas produced by cells of these two lines when injected into mice of the inbred strain from which the cells originated (*1*). Additional clones established from these original lines are designated by their NCTC clone numbers. Strains of tumor cells cultured from sarcomas produced by high- and low-line cells implanted in mice (*14*) are referred to as "high

and low lines after mouse passage." Of these, two strains are listed separately, strains 2432 and 2632. The distinctive properties of these two strains have been described (*14*). In addition, tests were made on two strains of fibroblasts recently isolated from subcutaneous adipose connective tissue of two additional C3Hf/He mice. Cells were cultured in T-60 flasks according to procedures previously described (*1–3*). The cells were grown on the glass floor of the flask.

Two types of culture medium were used: (1) a medium consisting of 40 percent horse serum, 20 percent chick-embryo extract (1:1.25), and 40 percent Earle's saline (*16*), or (2) chemically defined medium NCTC 109 (*17–19*) supplemented with 10 percent horse serum. Prolonged parallel transfers of the same line of cells in the two media have resulted in differences in cell characteristics that are ascribable to culture medium. That is, low-line cells have developed a higher transplantability *in vivo* following repeated passage in serum-supplemented chemically defined medium (*3*, *15*). However, glycolytic rates of similar lots of cells measured simultaneously in the two media are not significantly different. For this reason the results of activity measurements in the two media have been combined, unless otherwise noted. However, sensitivity of glycolysis to inhibition by *Podophyllum* fraction CC7E (*12*, *13*) is greater in the chemically defined medium, and it is necessary to take this into account in comparing the relative sensitivities of different cell lines to this inhibitor.

In the present study, in which we desired to compare a number of cell lines over considerable periods of time, only the anaerobic metabolism has been considered since, as Warburg has pointed out (*5*), aerobic fermentation is rather labile and easily modified by external conditions. For manometric measurements of glycolysis, the cells were changed to fresh culture medium and extra glucose and sodium bicarbonate added to give final levels of 0.016 M (0.3%) and 0.03 M, respectively. During filling of the vessels the cells were maintained at approximately 25° C., and the vessels were finally gassed with a mixture of 95 percent nitrogen and 5 percent carbon dioxide, with the thermostat at 37° C. The nitrogen-carbon dioxide mixture was passed, together with a trace of hydrogen, through a hot copper column (Kendall apparatus) to remove contaminating traces of oxygen. Rates of anaerobic acid production (at the expense of glucose) are expressed as μl. of CO_2 released from the medium per mg. calculated dry weight of cells per hour. Dry weights were calculated from packed-cell volumes, and were taken on the basis of preliminary average dry-weight determinations to be 10 percent of the measured centrifuge-packed volume of cells. Thus, aliquots of the same cultures used for metabolic determinations were spun uniformly in 3-ml. hematocrit tubes in an International horizontal centrifuge at an rcf of about 7000 $\times$ g, and each mm.^{3} of packed cells was taken as equivalent to 0.1 mg. of dry weight. This method provided a practical basis for making uniform comparisons of cell lines even when only small volumes of cells were available.

RESULTS

Anaerobic Glycolytic Properties of Cell Lines

Tables 1 and 2 present the anaerobic glycolytic activities, determined over a period of months, of the parent low (2049) and high (1742) lines, together with several clones derived from these. Two strains of recently isolated fibroblasts from subcutaneous adipose tissue of C3H mice are included for comparison. These latter strains had been cultured, respectively, for 2 and 5 months *in vitro*. While the 2-month-old strain had not been assayed for tumor-producing capacity, the 5-month-old strain failed to elicit any tumors when injected into 22 newborn mice and 1 irradiated adult mouse. These, and other determinations, have repeatedly indicated that low tumor-producing capacity [in terms of number of mice developing tumors after injection of tissue-culture cells, or, prolonged latent period for tumor appearance (*2*, *3*)] is associated with a relatively low glycolysis ($Q_{CO_2}^{N_2} = 30$ plus or minus 10) and high tumor-producing capacity with a relatively high glycolysis ($Q_{CO_2}^{N_2} = 100$ plus or minus 20).

The original high sarcoma-producing line (1742) and clones derived from it have consistently maintained high glycolytic quotients over a period of several years (*see* table 1). On the other hand, the original low sarcoma-producing line (2049) has been less uniform; in certain instances yielding cultures with significant elevations in $Q_{CO_2}^{N_2}$ (table 1). However, some cultures and clones of low line 2049 have retained relatively low glycolytic quotients (*see*, *e.g.*, clones 2445 and 2555 in table 1). It has been shown that both low- and high-line cells can be passed through a tumor generation *in vivo* without appreciable change in glycolytic characteristics. These low lines, while retaining such characteristics as low glycolysis, prolonged latent period *in vivo*, distinctive morphology, and failure to elicit a vascular reaction in the host, have, nevertheless, developed increased transplantability following mouse passage (*14*). In a few instances total conversions from low- to high-line properties, after mouse passage of line 2049 cells, have been noted. Two such derivatives of 2049 tumors, strains 2632 and 2432 (*14*), were examined in the present study. Both lines displayed high glycolytic quotients (table 1). In all instances, the tendency *in vitro* to change glycolytically has been from low toward high rather than in the reverse direction (tables 1 and 2).

Response of Tissue-Culture Lines to an Anti-Hexokinase Inhibitor

Because of previous work with mouse tumors *in vivo* (*6*, *7*, *9*) it was of special interest to determine the glycolytic response of high and low tumor-producing lines to specific inhibition of the anti-insulin type involving the hexokinase reaction (*10*, *11*). Preliminary studies showed that cells of the parent low tumor-producing line (2049) were 4 to 5 times more sensitive to inhibition by *Podophyllum* fraction CC7E (*12*, *13*) than were those of the parent high tumor-producing line (1742). Table 3 shows the relative glycolytic sensitivities of two clones derived from

TABLE 1.—Glycolytic activities of recently cultured fibroblasts, parent low and high sarcoma-producing lines, and several derived clones or strains

Cell line	Anaerobic glycolysis ($Q_{CO_2}^{N_2}$)	Date of determination
Recently isolated strains of fibroblasts from normal adipose tissue:		
2561 (Cultured 2 months *in vitro*)*	26†	20 Nov. '56
2661 (Cultured 5 months *in vitro*)*	39†	11 July '57
2049 (Parent low line)	28‡	4 Jan. '56
"	29†	16 Jan. '57
"	52‡	16 Jan. '57
"	72‡	12 June '57
Low-line clone 2445	28†	18 Oct. '56
"	54†	6 Dec. '56
"	37†	7 Feb. '58
"	24†	4 Mar. '58
Low-line clone 2555	21†	16 Jan. '57
"	20†	7 Nov. '57
"	35†	7 Feb. '58
"	30†	4 Mar. '58
Low (intermediate) line clone 2470	70†	18 Oct. '56
"	83†	20 Nov. '56
"	56†	17 Oct. '57
" transfer line #1	49†	14 Mar. '58
" " " #2	31†	14 Mar. '58
1742 (Parent high line)	125‡	6 Dec. '56
"	118†	16 Jan. '57
"	114‡	16 Jan. '57
High-line clone 2472	60†	18 Oct. '56
"	113†	20 Nov. '56
"	105†	7 Feb. '58
"	138†	11 Mar. '58
Strains cultured from tumor of 2049 low-line cells, and which resemble low-line cells:		
2314	27‡	4 May '56
"	48‡	25 May '56
2355	49‡	25 May '56
2576	43‡	30 Oct. '56
Strains cultured from tumor of 2049 low-line cells, and which resemble high-line cells:		
2632	106‡	21 May '57
2432	83‡	13 Sept. '56
"	83‡	18 July '56
"	85‡	18 Oct. '56
"	110‡	16 Jan. '57
2560 (Strain cultured from a tumor of 1742 high-line cells)	110‡	30 Oct. '56

*Strain 2561 not tested for tumor production; strain 2661 failed to produce any tumors when injected into 22 newborn mice and 1 irradiated adult mouse.

†Culture series in chemically defined medium plus 10 percent horse serum.

‡Culture series in Earle's saline plus 40 percent horse serum and 20 percent chick-embryo extract.

the parent low and high tumor-producing lines. It can be seen that the percentage inhibition with the low tumor-producing clone is greater than with the high tumor-producing clone. These results show that tissue-culture lines derived originally from one cell have developed wide differences in glycolytic sensitivity to inhibition by an anti-insulin (*i.e.*,

TABLE 2.—Summary (*see* table 1) of glycolytic activities of recently cultured fibroblasts and sarcoma-producing mouse tissue-culture cells

Cell lines	Glycolytic activity ($Q_{CO_2}^{N_2}$)		Number of cultures measured
	Average	Range	
Recently isolated fibroblasts	33	26–39	2
Parent low line (2049) and low clones derived from it	36	20–72	12
Clone 2470 derived from low line 2049	58	31–70	5
Parent high line (1742) and clones derived from it	110	60–138	7
Strains cultured from tumor of 2049 low-line cells, and which resemble low-line cells	42	27–49	4
Strains cultured from tumor of 2049 low-line cells, and which resemble high-line cells	93	83–110	5
Strain cultured from a tumor of 1742, high-line cells	110		1

TABLE 3.—Relation between anaerobic glycolytic capacity ($Q_{CO_2}^{N_2}$), sensitivity of glycolysis to inhibition by *Podophyllum* fraction CC7E, and average tumor latent period, in two tissue-culture clones, low-line clone 2445 and high-line clone 2472

Clone	Date	$Q_{CO_2}^{N_2}$	Percent inhibition of $Q_{CO_2}^{N_2}$ by CC7E	Average tumor latent period (*days*)*
2445	20 Nov. '56	46	42†	63
"	7 Feb. '58	37	60†	
2472	20 Nov. '56	115	3†	12
"	7 Feb. '58	105	0‡	

*Data from Sanford (*3*, chart 4, nonirradiated mice).
†*Podophyllum* fraction CC7E, 10 ppm.
‡*Podophyllum* fraction CC7E, 13 ppm.

anti-hexokinase) inhibitor. These differences in glycolytic rate, and in sensitivity to *Podophyllum* fraction CC7E, appear to be correlated with variations in certain tumor-producing characteristics. These relationships, which have already been reported (*2*, *3*), are illustrated in table 3 where data of Sanford (*3*, chart 4) for average tumor latent periods of the same two cell lines have been replotted. Thus, in cell lines maintained entirely *in vitro*, as in tumor lines maintained entirely *in vivo*, changes that can be interpreted as increases in certain tumor-producing properties are associated with losses in sensitivity of the cell glycolysis to specific inhibition of the anti-insulin type.

DISCUSSION

The present metabolic studies reveal two aspects of fundamental importance. The first, increase in the ability of tissue-culture cell lines to produce tumors, is associated with a marked increase in glycolytic

capacity ($Q_{CO_2}^{N_2}$) and, the second is associated with a marked *decrease* in the sensitivity of glycolysis to inhibition by an anti-insulin (*i.e.*, anti-hexokinase) inhibitor. These observations should be evaluated against the background of studies with tumors grown *in vivo* in which the degree of insulin:anti-insulin regulation of hexokinase reaction is critically involved in the malignant process (*6*, *7*, *9*). For example, most transfer lines of the Cloudman S91 mouse melanoma are very responsive to changes in host endocrine balance. After exposure to a stressful temperature, inhibition of tumor hexokinase is increased so that glycolysis is greatly lowered and tumor growth stopped. This inhibition of glycolysis can be largely reversed *in vitro* by insulin (*6*, *7*, *9*). Prolongation of stress results in adaptive change in the tumor-cell population such that strains of tumor cells arise which can grow fairly well in stressed animals, though more slowly than the original tumor in normal unstressed mice (*7*, *20*). Extreme insensitivity to regulation by host anti-insulin factors occurs in the long-transplanted Krebs-2 carcinoma in which glycolysis and growth are only slightly, or not at all, retarded by stressful conditions sufficient to markedly inhibit the glycolysis and stop the growth of S91 melanoma. Important from the standpoint of the present study is that mouse tumors *in vivo* show a range in sensitivity of glycolysis to inhibition by anti-insulins comparable to that occurring in the series of tissue cultures. In both cases increases in certain attributes of malignancy are associated with a lessened sensitivity of glycolysis to anti-insulin-type restraint. In this last connection the localization of insulin : anti-insulin control of hexokinase in the mitochondria by Hochstein (*10*, *11*) is of particular significance.

The studies of du Buy *et al.* (*21*, *22*) and Hesselbach and du Buy (*23*) had already shown that the mitochondria have an important function in cell glycolysis. Studies in higher plants have shown that these subcellular organelles possess properties of autonomous heredity so that in mutant forms they become the continuing cause of unique neoplasia (*24*). Mitochondria showing similar unique functional modifications can also be induced by virus action (*25*). In the series of tumors derived from the Cloudman S91 melanoma, which show gradations in glycolytic sensitivities to anti-insulin inhibitors, there are indications that the glycolytic changes involve the mitochondria (*6*, *9*, *11*, *21*). These mitochondrial considerations have important implications with respect to the changes *in vitro* of glycolytic characteristics in mouse tissue-culture cell lines.

Apparent conversions *in vivo* of low-line cells to high-line types may represent a process of rapid selection of pre-existing cell variants from an originally mixed low-line culture *in vitro* or an actual change *in vivo* due to induction or similar phenomenon. It should be noted that, while transformations involving shifts from low- to high-line characteristics appear to occur in cells grown entirely *in vitro* (*14*, *15*), many mouse passages of low-line cells have not resulted in changes in either glycolytic or tumor-producing characteristics. In this connection it is particularly

significant that the tendency to change glycolytically *in vitro* appears to be from low toward high rather than in the reverse direction.

Recently isolated non-tumor-producing fibroblasts and low-line sarcoma-producing clones (tables 1 and 2) had similar glycolytic capacities ($Q^{N_2}_{CO_2}$ = *ca.* 30). Obviously the capacity to form tumors depends on more than the mere attainment of such glycolytic levels, which are in the lower end of the tumor range and are similar to values reported by Warburg (*26*) for nonmalignant 8-day-old kidney explants. Warburg (*26*) has stated that completion of the malignant transformation *in vitro* involves not only greatly increased glycolysis (and uncoupling and/or fall in respiration and fall in catalase content) but also eventual and prolonged development of cellular resistance to immunochemical mechanisms *in vivo*. In this connection it seems likely that the metabolic pattern which develops shortly after explantation of normal cells to conditions *in vitro* also involves lowering of normal levels of hormonal restraint of hexokinase. Conversion to a state of demonstrable malignancy would require, *among other essential changes*, the appearance of *hereditary* reduction in the sensitivity of the cellular hexokinase to insulin: anti-insulin regulation so that growth-controlling levels of anti-insulin hormonal restraint could not be effectively imposed by the host after reimplantation of the culture cells. As previously indicated, there are reasons for believing that such a hereditary defect in susceptibility of the intracellular hexokinase to anti-insulin regulation could reside in the mitochondria.

Leslie *et al.* (*27*) have noted that certain human embryonic cells have a more active (tissue-culture) glucose metabolism *in vitro* than certain human carcinoma cells. They indicate that such data are contrary to the views of Warburg (*5*). This, however, is not actually the case, since Warburg (*4*, *5*) has shown that aerobic glycolysis of embryonal tissues results from damage incurred during exposure to conditions *in vitro*. Under conditions *in vivo* the high glycolytic potential of embryonal tissues is brought under hormonal restraint as the process of normal ontogeny proceeds, whereas the cancer cells remain chronically in a state of high glycolytic activity (*9*). The findings of Leslie *et al.* (*27*) that carcinoma cells can be more responsive to insulin than embryonal cells is in keeping with our own findings in mouse melanoma and mammary cancer (*9*).

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Chromocenters Resembling Sex Chromatin in Human Tissue-Culture Cell Lines [1]

SARA H. DEWITT, ALAN S. RABSON, FRANCES Y. LEGALLAIS, PASCO R. DEL VECCHIO, *and* RICHARD A. MALMGREN,[2] *Pathologic Anatomy Branch, Laboratory of Pathology, National Cancer Institute,[3] Bethesda, Maryland*

SUMMARY

Chromocenters resembling sex chromatin were studied in 12 human tissue-culture cell lines derived from both normal tissues and malignant tumors. All of these cell lines had low counts, and there was no correlation of the sex of the donor of the tissue from which the cell strain was derived with counts of chromocenters resembling sex chromatin of the cell-culture preparations. There was no significant difference in the counts in cell lines derived from normal human tissues and those derived from malignant tumors. This is in accord with the findings of others that cell strains derived from normal tissue are morphologically and biochemically similar to those derived from malignant tumors.—J. Nat. Cancer Inst. 23: 1089–1095, 1959.

BARR AND his associates described a special chromocenter in resting nuclei of normal female cells which they referred to as sex chromatin (*1*). Subsequently, a number of authors identified similar chromocenters in cells of human neoplasms (*2*). Orsi and Ritter (*3*) described sex chromatin in cultures of a cell line derived from a human mammary tumor, and Burlington (*4*) described such chromocenters in primary cultures of canine and feline kidney cells. In view of the morphologic and biochemical similarities of human cell-culture lines derived from different malignant tumors and from normal tissues (*5, 6*), we thought it of interest to attempt to identify chromocenters resembling sex chromatin in cells of a number of established cell-culture lines. The relative frequency of such chromocenters in 12 human cell lines has been determined.

MATERIALS AND METHODS

Cultures of 12 different cell lines have been studied and are listed in table 1 with the sex of the donors of the tissue of origin. The cell line,

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NCLP-2 derived in our laboratory from a cystosarcoma phyllodes of the breast, was grown in 20 percent human serum and 80 percent medium #199. All the other cell lines were grown in Eagle's basal medium with from 10 to 20 percent human serum. The cultures of NCLP-2 and LAC examined were from lines carried in our own laboratory. Cultures of D-189, D-227, and D-164 were obtained from Mr. Henry Orr, Laboratory of Chemical Pharmacology, National Cancer Institute. All the other cell lines were obtained from Microbiological Associates, Inc., including one group of D-189 cultures as noted in table 1.

Cultures were grown on coverslips in Leighton culture tubes. When confluent sheets were seen on the coverslips, the cultures were rinsed in saline and fixed for 6 to 24 hours in a modified Davidson's solution composed of 20 parts of formalin, 30 parts of 95 percent ethyl alcohol, 10 parts of glacial acetic acid, and 30 parts of distilled water. The coverslip preparations were then stained with a modified Feulgen technique (*7*) and counterstained with 0.01 percent light green in 95 percent ethyl alcohol.

The preparations were studied microscopically with an oil-immersion objective giving a final magnification of 1,455×. Cells with a Feulgen-positive body approximately 1 μ in diameter located against the inner surface of the nuclear membrane were counted as containing a chromocenter resembling sex chromatin. These chromatin structures were identified by their larger size in comparison with other chromatin particles in the nucleus adjacent to the nuclear membrane. The general shape of the chromocenter, although somewhat variable, was for the most part elongated and convex on the side away from the nuclear membrane. Occasionally the chromocenter was triangular or round in configuration (fig. 1).

Some mention should be made of the difficulties encountered in studies with tissue-culture cell lines. In spite of the fact that excellent fixation and staining can readily be accomplished with this type of material, the large amounts of coarsely granular chromatin in these cells make identification of chromocenters resembling sex chromatin difficult. In view of the known heteroploidy and aneuploidy of some tissue-culture cell lines (*8*), the presence of bodies resembling but often not typical of the sex chromatin body is not surprising. In our preliminary studies, great variations in counts by different observers of the same preparation, and even by the same observer in counts of a single preparation, were encountered. This was due to including small paranuclear chromatin granules that did not have the morphologic features of the sex chromatin as described by Barr (*2*). When more rigid criteria for identification of the body were employed, relatively consistent counts were obtained. Counts at various points on the same coverslip showed no significant differences. Counts made on cultures of the same cell line in active growth phases and in resting stages in maintenance medium also showed no significant differences.

It should be noted that in approximately 30 percent of cells containing

chromocenters resembling sex chromatin, more than one such body was found within a single nucleus (fig. 2). This was attributed to the polyploidy known to be present in many of these cell lines, and cells were counted only with respect to presence or absence of chromocenters resembling sex chromatin without regard to the actual number present in a single nucleus.

RESULTS AND DISCUSSION

The data have been summarized in table 1. The percentage of cells with chromocenters resembling sex chromatin is low in all the cell lines examined. This is not surprising since the established cell lines have the morphologic features of undifferentiated or anaplastic carcinoma cells (*5*, *6*). A number of authors have found low sex-chromatin counts in poorly differentiated or anaplastic tumors of humans (*2*, *9*).

There is no correlation of percentage of cells with chromocenters resembling sex chromatin and the sex of the donors of the tissues from which the cell lines were derived. All the cell lines derived from females had low counts. As Barr and Moore (*2*) have pointed out, however, in undifferentiated carcinoma in women these results are probably not the result of a chromosomal sex reversal but rather a reflection of the known chromosomal abnormalities in cell lines in continuous culture.

The results obtained with D-189 cell line are of interest. Although derived from the tissues of a male, this cell line had the highest counts of the 12 cell lines examined. In the first group of D-189 cultures, 27 percent of the cells had chromocenters resembling sex chromatin. A second group of D-189 cultures was subsequently studied, and the mean percent of cells with such chromocenters was 24. Nevertheless, these counts are not in the range usually found in normal female tissues, and these data do not conflict with the findings of others that, except for some teratomas, tumors of males do not have sex chromatin counts in the female range.

The D-189 cell line has been described in detail by Leighton and his associates and represents a line of altered or transformed cells that arose from a culture of foreskin fibroblasts (strain D), (*16*). Subsequently, 2 other lines of transformed cells were derived from cultures of the same foreskin fibroblasts (strain D) and designated D-164 and D-227.[4] As can be seen in table 1, both of these cell lines have counts lower than D-189 in spite of their common heritage. It is of further interest that cultures of D-189 obtained from another laboratory (Microbiological Associates, Inc.) had lower counts than cells of the same cell line grown at the National Cancer Institute, though the same growth medium was employed in both laboratories. This serves to emphasize the variability in even the same cell line maintained in different laboratories. In the course of multiple passages in several laboratories using somewhat different cultural methods, it seems reasonable that cell populations might be selected with different

[4] J. Leighton, personal communication.

TABLE 1.—Chromocenters resembling sex chromatin in human tissue-culture cell lines

Cell line	Tissue of origin	Reference	Sex of donor	Percent of cells containing chromocenters resembling sex chromatin*				Arithmetic mean of percent of cells with chromocenters resembling sex chromatin
				Observer I		Observer II		
				Slide 1	Slide 2	Slide 1	Slide 2	
HeLa	Carcinoma of cervix	(*10*)	Female	3	9	1	6	5
J-111	Leukemic blood	(*11*)	Female	3	7	6	4	5
NCLP-2	Malignant cystosarcoma phyllodes—breast.	†	Female	6	18	7	13	11
Chang-conjunctiva ‡	Conjunctiva—normal	(*12*)	Female	1	3	2	4	3
KB	Epidermoid carcinoma—floor of mouth	(*13*)	Male	6	8	6	6	7
Detroit 6	Sternal marrow—from patient with carcinoma of lung.	(*14*)	Male	12	22	13	16	16
LAC	Adenocarcinoma of lung	(*15*)	Male	2	3	3	7	4
Detroit 98	Bone marrow—normal	(*14*)	Male	2	4	2	5	3
Chang-liver‡	Liver—normal	(*12*)	Male	3	5	11	6	6
D-189 (1st group)	Altered cells from human foreskin—fibroblasts.	(*16*)	Male	27	27	26	29	27
D-189 (2d group)	"	(*16*)	Male	15	20	30	32	24
D-189 (Microbiological Assoc., Inc.)	"	(*16*)	Male	7	15	10	14	12
D-227	"	§	Male	11	17	18	11	15
D-164	"	§	Male	6	9	5	3	6

*One hundred cells counted per coverslip by each observer. Two coverslip preparations of each cell line were grown, fixed, and stained in the same way and designated, at random, as slide 1 and slide 2.

†A. Rabson, upublished data.

‡At the time these culture lines were initiated, Dr. Chang's sole objective was to obtain satisfactory cell cultures for virus isolation. Detailed records of the patients from whom the explants were obtained are not available. It is thought that the conjunctiva cell was derived from the conjunctiva of a young girl, and the liver cell from a middle-aged man.

§J. Leighton, personal communication.

chromosomal characteristics and corresponding differences in counts of chromocenters resembling sex chromatin. This would be in accord with the recent observations of Chu that different clonal populations of strain HeLa have significant chromosomal differences (*17*).

The possibility of contamination of one cell line by other cell-culture lines carried in the same laboratory or of the introduction of foreign cells with the serum in the culture medium has been suggested (*18*). Although these possibilities exist, whether this is true of the cultures studied cannot be evaluated and must be considered merely as one of the possibilities in any study of continuous-line tissue cultures.

Examination of the data in table 1 shows essentially similar counts in cell lines derived from normal tissues and from malignant tumors. This is in agreement with the observations of others that cell lines derived from normal tissues are morphologically and biochemically similar to those derived from malignant tumors (*5*, *19*).

Table 2 summarizes the information on the frequency of more than one chromocenter resembling sex chromatin per nucleus on four of the culture lines. Approximately one third of the cells with chromocenters resembling sex chromatin had more than one such body. In the cells with multiple chromocenters approximately 75 percent had 2, 10 percent had 3, and 15 percent had 4. It is most likely that these multiple chromocenters are associated with the polyploidy of the cells.

TABLE 2.—Cells with more than one chromocenter resembling sex chromatin per nucleus

Cell line	Percent of cells with more than one chromocenter resembling sex chromatin per nucleus*				Average percent of cells with more than one chromocenter resembling sex chromatin per nucleus
	Observer I		Observer II		
	Slide 1	Slide 2	Slide 1	Slide 2	
D-189 (1st group)	8	8	7	10	8
D-189 (2d group)	7	10	8	8	8
Detroit 6	2	5	3	5	4
NCLP-2	4	3	3	5	4

*One hundred cells counted per coverslip by each observer.

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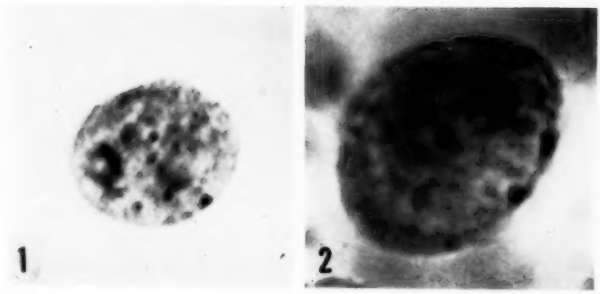
FIGURE 1.—Modified Feulgen-stained preparation from D-189 tissue-culture cell line showing one chromocenter resembling sex chromatin. × 1,600

FIGURE 2.—Modified Feulgen-stained preparation from D-189 tissue-culture cell line showing 2 chromocenters resembling sex chromatin. × 2,850



523596—59——27

Urate Excretion in Patients With Acute Leukemia [1, 2]

JAMES F. HOLLAND, *M.D.*, WILLIAM SHARPE, *M.D.*,[3] LOUISE M. MAMROD, *B.A.*, EDWARD DOWD, *Ph.D.*, *and* MARIAN HARTSOCK,[4] *Roswell Park Memorial Institute, Buffalo, New York, and Pennsylvania Hospital, Philadelphia, Pennsylvania*

SUMMARY

Urinary urate excretion was measured in patients with acute lymphocytic leukemia (ALL) and acute myelocytic leukemia (AML), while on a low-purine diet in the 5 days immediately before and the 10 days after onset of antileukemic treatment. As a group, ALL patients demonstrated increased excretion in the posttreatment period. Observations in individual patients revealed that this metabolic response was not essential to attainment of a complete remission of the leukemia, nor did its presence assure that remission would ensue. As a group, patients with AML who did not respond to treatment with remission or a precipitous fall in leukocytes had no significant change in their urate excretion. Attempts to identify those patients who attained remission, on the basis of their urate excretion before treatment, were not successful.—J. Nat. Cancer Inst. 23: 1097–1105, 1959.

DISORDERS OF uric acid metabolism associated with certain hematological diseases have long been known. More recently, urate excretion has been studied in the acute leukemias (*1–3*). Because uric acid is the predominant final oxidation product of physiological purines in man (*4, 5*), its excretion constitutes a partial indicator of nucleic acid catabolism. Increased excretion has been observed in the untreated state, and after antimetabolite treatment and steroid therapy in acute leukemia (*1–3*).

This report summarizes data on urate excretion obtained in the periods immediately before and after treatment of a group of patients with acute leukemia. Increase in urate excretion was observed after treatment of some but not all patients with acute lymphocytic leukemia (ALL). No increase in urate excretion after treatment occurred in patients with acute

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[3] Pennsylvania Hospital, 8th and Spruce Streets, Philadelphia 7, Penna.

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myelocytic leukemia (AML). Response to antileukemic therapy was not predictable from base-line uric acid excretion. No correlation was found between urate excretion after drug administration and therapeutic response in patients with ALL. These data extend the available information concerning urate excretion in acute leukemia (*1–3*), and emphasize that quantitative relationships between uric acid excretion, growth, and destruction of leukemic cells remain to be defined.

METHODS

Diagnosis of acute leukemia was made when a single type of immature leukocyte formed the predominant marrow component (*6*, *7*). AML was differentiated from ALL by morphological characteristics of aspirated marrow.[5]

AML was characterized by predominance of large mononuclear cells considered to be myeloblasts when the nucleus was round and central, occupied nearly all the cell, and had a delicate, evenly distributed reticular network. The cytoplasm was basophilic. Numerous nucleoli in the leukemic cell or the simultaneous presence of many promyelocytes or more mature granulocytic forms was accepted as corroborative evidence. Auer bodies were considered pathognomonic. In a few patients, the predominant cell was the promyelocyte, with fewer myeloblasts and a larger number of more mature forms. The clinical course of these patients was entirely consistent with that of other patients with AML.

ALL was characterized by a predominance of mononuclear cells considered to be lymphoblasts when the nucleus was round and occasionally indented or folded on itself slightly, when the nucleoplasm seemed more heavily condensed on the nuclear membrane, and when the nuclear chromatin was coarser than in myeloblasts. Clusters of cells showing varying maturity, from primitive large cells to smaller cells with more heavily aggregated chromatin clumps in the nucleoplasm and proportionately less cytoplasm, up to and including lymphocytes, were seen. Cytoplasmic pseudopodia, extensive vacuolization of the cytoplasm or nucleus, and apparent maturation of some cells toward lymphocytes were taken as corroborative evidence of ALL. The diagnosis of stem-cell leukemia is not used in this clinic. The diagnosis of unclassified acute leukemia was not made during the time of this study because it was believed possible ultimately to classify all patients. Two patients considered to have acute monocytic leukemia were observed during the time of this study and have been excluded from the analysis. All other patients with acute leukemia seen at this Institute from June 29, 1955, to July 12, 1957, were classified as AML or ALL. Classification was determined without reference to urate excretion. Patients with AML and ALL were omitted from the study only if urine collections were inadequate.

[5] Marrows were independently interpreted by the senior author and by Dr. Avery A. Sandberg, Roswell Park Memorial Institute. There was agreement in all instances.

Observations were made on hospitalized patients on a low-protein diet calculated by standard dietary tables to provide less than 70 mg. per day of exogenous purine compounds. Some specimens of urine were obtained before metabolic equilibrium could reasonably be anticipated in the pretreatment period, but no major difference in urate excretion attributable to this practice could be recognized.

Twenty-four-hour urine samples were collected at room temperature under toluene. Completeness of collection was determined by frequent reinstruction in collection technique to nurses and patients, by questioning, and by comparison of volume with the usual urinary output for a particular patient. These criteria appeared to be more valuable than the creatinine determinations that were performed on some specimens. Doubtful collections were discarded.

Urinary urate was determined by differential spectrophotometry before and after addition of uricase (*8, 9*).

Urate excretion is given with reference to body surface area because of the applicability of this standard to a combined series of adults and children, and to other metabolic characteristics (*10, 11*). Information is presented for 15 days only, divided into three 5-day periods, one immediately preceding the onset of therapy and the first two 5-day periods immediately succeeding. The mean urate excretion for all complete specimens and mean leukocyte count for all observations made in each period were calculated for each patient. In addition, the mean daily urate excretion for each type of leukemia is presented graphically.[6] Average values are computed from a differing number of observations dependent on the valid specimens available for any one day. The determination of remission status was made in accordance with a recently published definition (*12*).

Urate excretion was measured in 4 healthy children in 1 family on a low-purine diet for either 1 or 2 days before collection. Urate excretion was measured in 4 healthy young adults after 3 days on the same calculated low-purine diet used for patients. The data for adults are similar to normal values previously published (*1*) (table 1).

RESULTS

The patients studied are presented in table 2. During our observations a study of the therapeutic effect of combined 6-mercaptopurine (6-MP) and methotrexate (Mtx), each in conventional dose range, was in progress (*6*). Some patients received 7.5 mg. Mtx every 3d day, while others, chosen randomly, received 2.5 mg. Mtx daily. In each combination regimen, 6-MP was administered at a dose level of 3 mg. per kg. per day. A few patients in a subsequent study were assigned to treatment with one or the other antimetabolite alone. Corticosteroids

[6] Reprints will include data for each patient in tables 2, 3, and 4.

TABLE 1.—Twenty-four-hour urinary urate excretion in normal subjects

Subject	Age	Sex	Height (*cm.*)	Weight (*kg.*)	Surface area (*square meter*)	Twenty-four-hour urate excretion	
						Total mg.	Mg./ square meter
			Healthy siblings:				
I	4	F	106	16	0. 95	199	208
II	6	F	122	25	1. 27	296	233
III*	8	M	127	25	1. 30	244	188
IV	10	M	160	52	1. 52	461	303
			Healthy adults:				
V	22	F	159	47	1. 45	390	269
VI†	28	F	161	59	1. 61	390	242
VII	31	M	183	82	2. 02	435	215
VIII	31	M	175	73	1. 87	486	260

*Mental deficiency.
†Two months pregnant.

were used nonelectively in patients with bleeding or with life-threatening complications. In the 3 patients treated by a single antimetabolite alone, for whom pre- and posttreatment data are available, no differences in the pattern of urate excretion can be appreciated from the group treated with combined antimetabolites. Since the number of patients in any treatment group is small, all treatment groups have been considered as one.

Among 10 patients with ALL, only 1 had not previously received antileukemic treatment. At the time of onset of treatment in this study, however, no patient was in remission. Three of 10 patients sustained substantial hematologic improvement, indicating that they were not in a refractory stage of advanced ALL. In the first 5 posttreatment days, 9 of the 10 patients had a decrease in peripheral leukocyte count. In the second 5-day period, leukocyte counts continued to fall or remained depressed at leukopenic levels in all but 1 patient.

Text-figure 1 shows the mean daily urate excretion, with 95 percent confidence limits for 10 patients with ALL. The pretreatment level exceeds the normal. There is a rise in urate excretion that reaches a maximum on the 5th posttreatment day, and then returns to pretreatment levels. When the excretion in the first 5 days post treatment is compared with the 5 days pretreatment, there is a difference with statistical significance of $0.05 < P < 0.10$ when analyzed by Student's "*t*" test with appropriate adjustment for inequality in variances. Other differences among the 5-day periods are not significant ($P > 0.10$).

The amount of change of urinary uric acid in the first 10 days of antileukemic therapy was not necessarily a sign of oncoming remission. Patients 6, 7, and 8 (tables 2 and 3) eventually attained remission status (*12*), and study of their individual urate excretions revealed, respectively, a probable increase, a definite increase, and no appreciable change. Among individuals who did not respond to treatment with a remission,

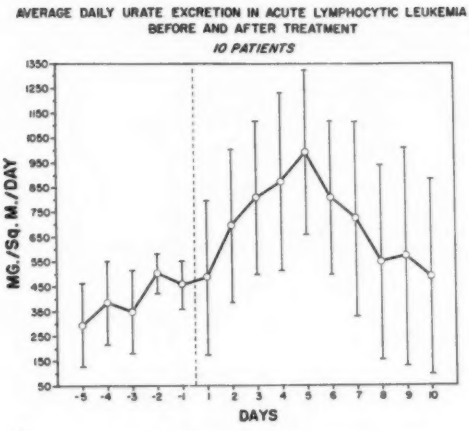
TABLE 2.—Patient identification

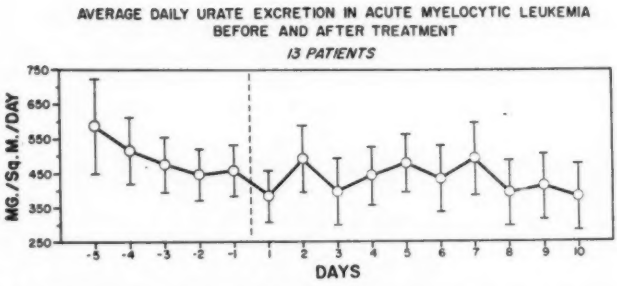
Patient	Unit No.	Age	Sex	Weight (*kg.*)	Height (*cm.*)	Surface area (*square meter*)	Treatment	Remission	Remarks†
					ALL				
1	81, 592	4	F	16	—	0. 64*	6-MP–Mtx	0	R–6-MP–Mtx
2	88, 474	4	F	17	104	0. 69	6-MP–Mtx	0	A
3	86, 446	6	F	19	96	0. 68	6-MP–Mtx	0	R–S
4	84, 378	8	F	25	—	0. 94*	6-MP–Mtx	0	R–S
5	82, 826	8	M	27	—	0. 98*	6-MP–Mtx	0	R–S,X,TEM
6	87, 994	13	F	46	157	1. 43	6-MP–Mtx	Partial	6-MP,A,a
7	87, 916	14	M	55	165	1. 58	6-MP–Mtx	Complete	B
8	84, 964	14	M	41	142	1. 42	6-MP–Mtx	Complete	S
9	90, 389	38	F	84	160‡	1. 86	6-MP	0	C
10	89, 620	38	F	85	152‡	1. 81	Mtx	0	
					AML				
11	84, 017	5	M	20	—	0. 78*	6-MP–Mtx	0	C
12	86, 016	14	M	43	175	1. 49	6-MP–Mtx	0	X
13	90, 516	22	F	44	165	1. 47	6-MP–Mtx	0	
14	84, 751	26	M	60	173	1. 70	6-MP–Mtx	Complete	R–6-MP
15	90, 994	33	F	56	160	1. 57	6-MP	0	
16	84, 981	41	F	74	175	1. 89	6-MP–Mtx	0	
17	84, 772	50	M	68	170	1. 77	6-MP–Mtx	0	
18	90, 333	54	M	61	180	1. 79	Mtx	0	
19	89, 951	54	F	78	157	1. 79	Mtx	0	
20	90, 680	55	F	70	175	1. 84	6-MP–Mtx	Complete	
21	84, 608	59	F	70	165	1. 76	6-MP–Mtx	0	D
22	88, 615	64	M	62	180	1. 79	6-MP	0	
23	90, 455	70	M	68	167	1. 76	6-MP–Mtx	0	E

*Surface area calculated using weight-surface area nomogram (*11*).

†R = Remission and relapse after drug named when previously given. Drugs listed without R denote that remission did not occur. S = prior corticosteroid in conventional dose. X = prior localized X-ray treatment. A = maintenance prednisone dosage recently increased to 30–40 mg./day. a = Local irradiation, bone, 53 r, days 1 and 2 before antimetabolites. B = prednisone begun on 5th day of antimetabolite therapy. C = steroids stopped 7 days before antimetabolite therapy. D = maintenance cortisone, 75 mg./day on antimetabolite days 1 through 7, 20 units ACTH gel on days 8 through 10. E = died from sepsis. Had pancytopenia and hypocellular marrow aspiration in which leukemic cells could not be identified after drug administration. At autopsy, no pathognomonic evidence of leukemia.

‡Height estimated from memory.





TEXT-FIGURE 1.—Mean and 95 percent confidence limits are shown.

TABLE 3.—Average 24-hour urate excretion per square meter and leukocyte count before and after treatment of ALL patients

	Before treatment		After treatment			
	5 days		First 5 days		Second 5 days	
Patient	Leukocytes (*per mm.*³)	Urate (*mg.*)	Leukocytes (*per mm.*³)	Urate (*mg.*)	Leukocytes (*per mm.*³)	Urate (*mg.*)
1	1, 380	367	1, 275	584	600	569
2	4, 600	558	2, 525	940	1, 675	577
3	19, 800	480	3, 920	877	183	1, 820
4	75, 250	632	2, 880	1, 613	775	876
5	5, 000	546	1, 750	414	2, 500	406
6	14, 500	592	7, 000	745	5, 100	595
7	4, 225	273	3, 450	—	3, 375	683
8	2, 500	357	1, 000	344	1, 375	404
9	45, 500	378	226, 000	357	240, 000	—
10	44, 700	433	26, 000	722	1, 760	849

however, 5 demonstrated increased urate excretion concomitantly with fall in leukocyte count, and 1 (patient 5) failed to do so.

Among 13 patients with AML, 9 of whom had been previously untreated, the average pretreatment urinary urate was similar to that of patients with ALL and substantially higher than normal. The average leukocyte counts did not change appreciably in the first 5-day period (table 4). In text-figure 1, it is apparent that no material change in urate excretion occurred in the pre- or posttreatment periods for the group. Unfortunately, patients 14 and 20, who subsequently attained complete remission status, are represented by only 5 and 1 posttreatment urine specimens, respectively, so that no statement can be made concerning predictability of response to chemotherapy from the posttreatment urate data.

TABLE 4.—Average 24-hour urate excretion per square meter and leukocyte count before and after treatment of AML patients

	Before treatment		After treatment			
	5 days		First 5 days		Second 5 days	
Patient	Leukocytes (*per mm.*^{3})	Urate (*mg.*)	Leukocytes (*per mm.*^{3})	Urate (*mg.*)	Leukocytes (*per mm.*^{3})	Urate (*mg.*)
11	13, 200	553	10, 000	311	4, 000	304
12	167, 000	608	169, 000	655	80, 400	591
13	32, 050	519	19, 500	478	19, 250	—
14	35, 250	419	45, 500	486	24, 000	—
15	6, 850	599	6, 100	—	5, 450	—
16	36, 900	257	42, 250	310	29, 300	297
17	79, 250	—	87, 500	374	—	—
18	3, 400	472	2, 600	515	1, 575	442
19	1, 450	—	2, 100	308	1, 550	377
20	61, 000	358	47, 800	359	41, 000	—
21	8, 750	563	8, 250	394	2, 500	440
22	20, 000	519	23, 800	474	12, 300	517
23	13, 000	271	13, 400	222	10, 550	—

DISCUSSION

The patients who entered the study, although each was in an active progressive phase, were not representative of untreated acute leukemia. Several selecting factors were operative. Some patients were excluded who could not collect urine specimens reliably because of young age or severity of illness. Only those patients who could be hospitalized conveniently and who survived long enough to be treated with antimetabolites were included. Many individuals had had prior steroid or antimetabolite drugs and in a few cases maintenance steroid compounds were being administered.

The base-line average urate excretions for patients with AML and ALL are approximately the same. A significant difference between the two types of disease is seen ($P < 0.05$), however, when one considers

urate excretion in the first 5 posttreatment days. The failure to observe increased urate excretion, after treatment, in AML is at variance with findings of Sandberg *et al.* (*1*). No precipitous fall in leukocyte count occurred in the patients with AML whom we studied, however, which may account for the discrepancy.

There is a difference in the age span of the 2 groups. The urate excretion pattern is not apparently age dependent, however. The 2 children with AML did not increase their urate excretion. Although 5 of the 7 children with ALL who were studied in the first 5 posttreatment days increased their urate excretion, patients 5 and 8 failed to do so.

Within the ALL group no consistent difference was noted among those patients who subsequently responded with hematological improvement and those who failed to respond. Thus, patient 8 enjoyed 29 months of complete remission, but failed to modify his urate excretion during the period considered here (or during several weeks thereafter). Contrariwise, patient 4 manifested a major change in urate excretion with marked regression of leukemic infiltrates and striking but evanescent hematological improvement; 36 days after drug administration, she was again in frank leukemic status. One may surmise that increased uric acid excretion after treatment is related to leukemic cellular disappearance from blood and tissues, but that these phenomena are not necessarily indicative of oncoming remission status. (Patients 1, 2, 3, 4, and 10.)

Acute lymphocytic leukemic cells apparently provide a pool of precursors of urinary urate. It is also possible that extraleukocytic pools of urate precursors and body pools of urate contribute to the increased excretion of urate for the several days immediately following the institution of chemotherapy. Increased body pools of urate have been demonstrated in some patients with chronic myelocytic and chronic lymphocytic leukemia (*13*, *14*). In some patients with ALL, elevated concentrations of urate in serum before treatment suggest that the extracellular urate pool may be high. Pathways of purine metabolism uninvolved in nucleic acid catabolism may have been substantially affected by the drugs administered. Our data do not indicate the proportionate contribution of partial body pools to the increased urate excretion. Nonetheless, it is probable that urate excretion is related to nucleic acid catabolism in some quantitative manner. A leukemic individual with low urate excretion may have rates of leukemic cell production and destruction different from a patient with high excretion.

Early in the study our results suggested that individuals with AML who had a pretreatment urate excretion of less than 450 mg. per square meter per day were more likely to achieve remission status. When the data of Sandberg *et al.* (*1*) and Haut *et al.* (*15*) are recalculated, however, and added to our own cases, the suggested difference is not convincing. The pretreatment urate excretion in patients with ALL who attained remission status was indistinguishable from the rest of the ALL group.

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Hormonal Influences on the Growth of a Transplantable Mammary Adenocarcinoma in C3H Mice [1,2,3]

BARBARA B. JACOBS[4] *and* ROBERT A. HUSEBY,
Department of Surgery, Indiana University Medical Center, Indianapolis, Indiana, and the Department of Surgery, University of Colorado School of Medicine, Denver, Colorado

SUMMARY

Propylthiouracil administration produced a marked body weight loss in C3H mice and a significant depression of the growth rate of the DRV mammary adenocarcinoma in these hosts. An equal degree of inanition attained by dietary restriction produced comparable tumor growth suppression. Mice made hypothyroid by thyroid-lethal doses of radioactive iodine did not lose weight and the tumor growth rate was unaffected in such hosts. The DRV mammary tumor grew equally well in intact males and intact females and more rapidly in castrate than in intact mice of either sex. The administration of a nontoxic dose of diethylstilbestrol to castrated female mice decreased the rate of growth of the tumor, while the administration of prolactin to ovariectomized diethylstilbestrol-treated females enhanced tumor growth. However, lactation *per se* did not influence the growth rate of the tumor. Although the DRV tumor appears to be responsive to one or more pituitary hormones, it does not appear to be hormone dependent since it grew progressively in hypophysectomized hosts.—J. Nat. Cancer Inst. 23 : 1107–1121, 1959.

ALTHOUGH THE importance of hormones in the genesis of spontaneous mammary cancer in mice is well established, hormone dependency of such tumors when they have developed has not been extensively investigated. The picture, which has been most generally described, is one of progressive growth, with hormonal alterations seldom causing significant changes in growth rate. For example, Gardner (*1*) described spontaneous mammary tumors that continued to grow almost as rapidly after hypophysectomy as similar tumors growing in intact mice. For a review of the older literature, *see* Shimkin (*2*).

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[3] Data dealing with PTU and I^{131} were included in a thesis submitted in partial fulfillment of requirements for the Ph.D. in Zoology at Indiana University. This part of the work was done at the Indiana University Medical Center and was supported in part by an Institutional Grant from the American Cancer Society, Inc., and in part by the Alexander and Margaret Stewart Trust Fund.
[4] *Present address:* Department of Breast Surgery, Roswell Park Memorial Institute, Buffalo, New York.

More recently, with increased interest in the hormone dependency of tumors, a number of instances of hormonal effects on the growth of mammary tumors in mice have been recorded. The most striking of these is a description by Foulds (*3*, *4*) of several spontaneous mammary tumors, which grew progressively during pregnancy and regressed either fully or partially after parturition only to reappear during the next period of gestation. It was noted that some of these tumors eventually lost their hormone dependency and continued to grow progressively regardless of the reproductive state of the host. Foulds (*5*) also has described several spontaneous tumors which, when transplanted, grew only in female mice or in males that were estrogenized. After several passages, however, these tumors grew in untreated males as well as in females. Mühlbock (*6*) found that although ovariectomy had little effect on the growth of spontaneous mammary tumors in C3H mice, estrogen administration did cause a significant inhibition of growth. Although the early reports of the effects of testosterone administration on the growth rate of spontaneous mammary tumors in C3H mice were essentially negative (*7*, *8*), more recently Foley (*9*) has reported that the administration of 2 orally active androgens retarded the growth of the C3HBA mammary adenocarcinoma. Liebelt (*10*) observed that lactation after pregnancy or induced by pituitary isografts slowed the growth of both spontaneous and transplanted tumors in RIII mice.

The administration of either adrenal corticoids or ACTH has been reported by several investigators to retard the growth of transplanted and spontaneous mammary tumors in mice, as well as to induce massive necrosis in well-established tumors (*11*). These effects persisted even though the mice were being treated simultaneously with somatotropic hormones, thus circumventing the weight loss that is usually incident to treatment with large doses of corticoids (*12*, *13*). It also has been reported (*14*) that even though the growth of the primary transplant is slowed, there is an increased incidence of metastases, so that the tendency for tumor cells to embolize and for these emboli to grow to macroscopic size is enhanced.

The development of spontaneous mammary tumors in females of several susceptible strains of mice has been prevented or markedly reduced by the administration of thiourea or thiouracil (*15*, *16*), but the possible dependency of mammary tumors of mice on thyroid function has not been investigated. We therefore initiated the present studies with an investigation of the effects of hypothyroidism (*17*) on the growth rate of a transplantable mammary adenocarcinoma.

METHODS

Inbred mice derived from the Z(C3H)/Bittner line were used, and the tumor, designated as DRV, originated as a spontaneous mammary adenocarcinoma in a female of this strain. Serial transplants of the tumor

were made by suspending 1 part minced tumor tissue in 10 parts saline and injecting 0.2 ml. of the suspension subcutaneously, ½-cm. cephalad to the base of the tail. The tumor routinely reached a weight of 1 to 2 gm. 2 to 3 weeks after inoculation. The tumor has maintained a well-differentiated glandular morphology (fig. 1) as well as a consistent rate of growth throughout 105 serial transfers over a period of 5 years.

In the experiments recorded in tables 1 and 2, a somewhat more complicated preparation of tumor inoculums was used in an attempt to obtain greater uniformity in tumor size. However, with this method the tumor grew more slowly (approximately 7 weeks being required for the tumors to reach an average weight of 1–2 gm.) and the growth rate was actually less uniform than that observed with the routine method of preparation. We therefore used the simpler technique in the remainder of the experiments.

Inoculations always were made alternately into mice of the various groups within any given experiment to distribute equally differences in cell viability and cell concentration that might occur during the period required for the injections to be completed. In each experiment the mice of the various groups were matched well with regard to age, weight, and littermate distribution.

Mice were rendered hypothyroid by the administration of either propylthiouracil [5] (PTU) or I^{131}. In the first instance, PTU was added to powdered Purina laboratory chow to a final concentration of 1 percent. After the mice had ingested this diet for 4 to 12 weeks, histological examination of the thyroids routinely revealed the extensive hypertrophic and hyperplastic changes in the epithelium which are characteristic of the hypothyroidism induced by goitrogenic drugs. In the second instance, 200 μc of I^{131} in 0.05-ml. KI-saline solution was administered intraperitoneally 1 month prior to tumor implantation. In agreement with the findings of Gorbman (*18*), serial sections of the thyroid-parathyroid region of mice receiving this treatment revealed scar tissue, intact parathyroids, and only an occasional thyroid follicle.

Castrations, when performed, were done 1 to 5 days prior to tumor implantation. The stilbestrol (diethylstilbestrol) was incorporated into a semisynthetic diet (*19*), 0.2 γ per gm. of food, and the controls received the same diet minus stilbestrol. Mice ingested 2 to 2.5 gm. of this diet per day (*20*). These diets began on the day the tumor was inoculated and were continued throughout the course of the experiment.

The prolactin [6] (NIH-SP-1) used is a highly purified sheep-pituitary preparation estimated to contain 15 I.U. per mg. One mg. of this hormone was injected intraperitoneally on the day after tumor inoculation, and then daily throughout the remainder of the experiment.

In several experiments, the dry as well as the wet weights of the tumors were recorded. Since the relationship of dry weight to wet weight was

[5] Supplied by Dr. S. M. Hardy, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

[6] The prolactin was received from the Endocrinology Study Section, National Institutes of Health.

the same in the animals of the various experimental groups, only the fresh weights are recorded herein.

RESULTS

The initial experiment was designed to study the effect of various periods of hypothyroidism on the rate of growth of the DRV tumor, hence the PTU feeding was initiated at 3 different times in relation to tumor implantation. Animals in group I were started on the diet 31 days prior to implantation, those in group II on the day the tumor was inoculated, while those in group III did not begin to ingest PTU until 23 days after tumor inoculation, at which time many had small but palpable tumors. All animals were killed 51 days after tumor inoculation. As indicated in table 1, the mean tumor weight for group I and group II was similar, while that for group III was slightly though not significantly greater statistically. However, the mean tumor weight of the control group of animals was significantly greater than that of the 3 experimental groups. A marked loss of weight occurred in all groups of animals ingesting PTU.

TABLE 1.—Tumor and body weights in mice receiving 1-percent PTU diet for three different periods in relation to time of tumor implantation

Treatment of host	Number mice	Total days on PTU	Mean tumor weight (*gm.*)	Mean body weight (*gm.*)
PTU-I	14	82	0. 41	16. 6
PTU-II	25	51	0. 39	17. 1
PTU-III	17	28	0. 78	18. 1
Control	30	0	1. 79*	25. 7

*Difference between means of control and combined experimental groups statistically significant ($P = <0.05$).

In an attempt to evaluate the relative importance of weight loss and of hypothyroidism *per se*, the effect of hypothyroidism induced by PTU ingestion was compared with that incident to treatment with a thyroid-lethal dose of I^{131}, and the results are recorded in table 2. Again, rendering the animals hypothyroid by means of PTU prior to tumor inoculation (group I) was no more effective in reducing the growth rate of the tumor than the initiation of treatment was at the time of inoculation (group II). In both of these groups the mean body weight was lowered significantly, most probably as a direct result of the decreased consumption of food, and the animals were essentially anestrus throughout the entire period of the experiment. In contrast, the animals rendered hypothyroid by the administration of I^{131} did not lose weight and were not anestrus; the mean tumor size was not reduced. It was noted that a significant pituitary

enlargement occurred in the animals treated with I^{131}, but not in those treated with PTU.

TABLE 2.—Effect of treatment with PTU and I^{131} on tumor and organ weights and estrous frequency

Treatment group	Number of mice	Mean food intake/ day (*gm.*)	Mean body weight (*gm.*)	Mean tumor weight (*gm.*)	Positive estrous smears* (*percent*)	Mean thyroid weight (*mg.*)	Mean pituitary weight (*mg.*)
PTU-I†	11	2. 0	15. 2	0. 17	1. 3	8. 5	2. 7
PTU-II†	24	2. 1	17. 4	0. 09	0. 0	6. 2	2. 5
I^{131}‡	39	—	26. 5	0. 86§	10. 6§	—	4. 2§
Control	32	2. 7	24. 1	0. 80§	16. 9§	2. 6	2. 5

*Estrous vaginal smears were taken on days 71 to 81 of the experiment.
†These groups correspond to the same designation as in table 1.
‡200 μc injected 1 month prior to tumor implantation.
§Values are statistically significantly greater than those for PTU-fed groups ($P = <0.01$).

To establish more firmly the importance of undernutrition in altering the growth rate of this tumor, a third set of experiments was undertaken in which the effectiveness of underfeeding was compared with that of PTU ingestion. The amount of food made available to the mice on the restricted diet was reduced to a degree so that they would lose weight at the same rate as the mice ingesting the PTU diet. In the first several attempts, we were unsuccessful in reducing the weight of the restricted-diet mice to as great an extent as desired; nevertheless, in all of these experiments the mean tumor weight in the restricted animals was less than noted in the fully fed animals. The experiment recorded in table 3 represents the data obtained in a single experiment in which we were fairly successful in our attempt to match weights. From these results, it is evident that the growth rate of this tumor was retarded considerably by undernutrition, and it appears most probable that the effectiveness of feeding the PTU diet resided entirely in the fact that the animals voluntarily restricted their food consumption when they ingested this diet.

A series of experiments were then carried out to establish, if possible, the mechanism by which undernutrition brings about a reduction in the rate

TABLE 3.—Comparative effect of diet restriction and of PTU consumption on tumor growth*

	Females			Males		
Diet†	Number mice	Mean body weight (*gm.*)	Mean tumor weight (*gm.*)	Number mice	Mean body weight (*gm.*)	Mean tumor weight (*gm.*)
PTU-*ad libitum*	20	18. 7	1. 25	30	21. 3	1. 50
Control-restricted	21	18. 1	1. 34	32	19. 4	1. 18‡
Control-*ad libitum*	16	25. 5	1. 76	40	28. 6	1. 83

*In the experiments recorded in tables 3 to 6, the animals were killed 14 to 16 days after tumor inoculation.
†Diets were initiated 1 month prior to tumor implantation.
‡The difference between this group and the control males is statistically significant ($P = <0.004$).

of growth of this tumor. It is well established that inanition in rodents reduces certain areas of pituitary function which result in a secondary gonadal hypofunction (*21*, *22*). Although reduced gonadal function might be responsible for slowing the tumor growth, the observations that the growth rate was essentially the same in intact male and intact female animals and that undernutrition was equally as effective in retarding tumor growth in both sexes suggest that a pituitary factor is involved directly rather than via gonadal mediation. To investigate this possibility, animals of both sexes were castrated in order to increase the pituitary secretion of at least the gonadotropins. As recorded in table 4, the removal of the gonads from animals of either sex resulted in a significant increase in the growth rate of the tumor. Treatment of castrate female animals with a moderate dose of stilbestrol reduced the growth rate of the tumor to that seen in intact female animals, but treatment of castrate males with the same amount of stilbestrol was without effect. It should be noted that the final body weights of these mice were not affected by the stilbestrol treatment.

TABLE 4.—Effect of castration and of stilbestrol ingestion on tumor growth in male and female mice

Type host	Number mice	Mean tumor weight (*gm.*)	Standard deviation
Intact female	62	1. 82	1. 48
Intact male	58	1. 59	1. 24
Castrate female	55	3. 22*	2. 62
Castrate male	58	2. 79*	2. 13
Castrate female and stilbestrol	62	1. 87†	2. 08
Castrate male and stilbestrol	72	2. 35	1. 69

*Value statistically significantly greater than that for intact mice ($P = <0.01$).
†Value statistically significantly less than that for untreated castrate mice ($P = <0.05$).

These latter observations would support the thesis that the pituitary was exerting a direct effect upon tumor growth if it could be shown that pituitary function in the male is more resistant to suppression by stilbestrol than in the female. As has already been reported (*23*), male mice of the BALB/c strain remain fertile and show only a minor reduction in seminal vesicular weight when maintained on this stilbestrol-containing diet. Similarly, we observed that male mice of the C3H strain maintained on this diet showed little if any change in testicular morphology. However, in the ovaries of female animals that had been ingesting the diet for 3 to 6 weeks, the interstitial cells were uniformly small, but had scanty cytoplasm and a characteristic "wheel-cell" nuclear pattern. Although during this short period of estrogenization the follicles and corpora lutea did not appear to have been greatly affected, the definite change in the interstitial cells strongly suggests a reduced gonadotropic stimulation. To test this possibility further, 48 weanling male and female mice were castrated, and 2 ovaries from weanling females were then transplanted into the left axillary tissues of each animal. Four weeks later, some of the animals were placed on the stilbestrol-containing diet for periods of 3 to 6 weeks,

while the others were maintained on a nonestrogen-containing diet. Histological examination of the ovaries transplanted into female mice ingesting stilbestrol revealed that the interstitial cells were, by and large, reduced to the dense "wheel-cell" pattern characteristic of a low gonadotropic stimulation (fig. 2). In the ovaries transplanted into male mice ingesting the same stilbestrol diet, the picture was significantly different in that the interstitial cells still possessed a considerable amount of cytoplasm and their nuclei did not have the "wheel-like" chromatin pattern (fig. 3). Although the interstitial cells of the ovarian transplants in these stilbestrol-treated male mice were not as large and as "degranulated" as those in ovaries from untreated males, their morphology was similar to that seen in untreated female mice (fig. 4). These findings would appear to support the idea that pituitary release of gonadotropins in male mice is more resistant to suppression by stilbestrol than in female mice.

The next logical step would be to determine which pituitary factor is involved in stimulating the rate of growth of this particular tumor, and our first experiment yielded suggestive results. Female mice were ovariectomized and placed on the stilbestrol-containing diet. On the day after tumor inoculation, approximately one half of the animals were begun on daily intraperitoneal injections of 1 mg. of purified sheep-pituitary prolactin. As can be seen in table 5, the mean tumor weight of the prolactin-treated females was significantly greater than that of the nonprolactin-treated animals. As far as we could determine, treatment of intact C3H female mice that were ingesting the stilbestrol-containing diet with prolactin was without effect upon the appearance of the ovarian interstitial cells. Although the growth rate of this tumor was increased by prolactin administration, growth was no more rapid in female mice that were actively nursing their first litter than in virginal females of comparable age (table 6).

TABLE 5.—Effect of treatment with a purified prolactin preparation on tumor growth in castrate female mice ingesting stilbestrol

Treatment of host*	Number mice	Mean tumor weight (*gm.*)	Standard deviation
Stilbestrol	31	1. 12	0. 98
Stilbestrol and prolactin	35	2. 35†	1. 22

*The mean body weights of the mice in these 2 groups were similar.

†Difference between mean tumor weights of the 2 groups is statistically significant ($P = <0.01$).

TABLE 6.—Lack of effect of lactation on the growth rate of the DRV tumor

Type host	Number mice	Mean tumor weight (*gm.*)	Standard deviation
Lactating	40	1. 30	1. 50
Nonlactating	46	1. 44	1. 67

It was, however, interesting to find that this hormone-responsive mammary tumor was not completely dependent upon pituitary hormones. We observed that tumors which were implanted into 8 untreated hypophysectomized male hosts grew progressively and at a rate similar to that in 9 intact male hosts whose food intake was restricted to a degree that mice in both groups had comparable body weights. The final mean tumor weights for the hypophysectomized mice and those on a restricted diet were 2.22 and 2.12 gm., respectively.

It also should be noted that, as far as we could tell from histological examination, none of the endocrinological manipulations used in these studies altered the rather well-differentiated glandular pattern of this adenocarcinoma, though several of these manipulations did affect materially the morphology of the normal mammae. In addition, the growth rate of the tumor did not appear to be correlated with the type of response seen in the hosts' normal mammary epithelium. Thus, ovariectomy, which affected regressive changes in the normal epithelium, stimulated the tumor growth; stilbestrol administration to castrated females stimulated normal glandular epithelium but suppressed tumor growth. However, additional treatment with prolactin stimulated both the glandular epithelium and the growth rate of the tumor.

DISCUSSION

Due to the relative newness of the concept that certain malignant neoplasms are not completely autonomous, a unified descriptive terminology does not exist. The adjective "dependent" came to be applied to such neoplasms as a result of the observed regressions that occurred in clinical material after hormonal alterations; for it appeared that continued growth of such tumors was, at least temporarily, dependent on specific stimulating influences arising from the host. Clearly, however, the terms "autonomous" and "dependent" are not necessarily antonyms, for the former connotes *self-government* or a complete freedom from external control, while the latter implies a *reliance* upon external factors. Alterations in the rate of growth of neoplasms or in the degree to which they function might well represent examples of responsiveness of tumors to outside influences without necessarily suggesting that such tumors are "reliant" upon such stimuli for their continued growth. The tumor used in the present studies seems to be an example of such a phenomenon, for its rate of growth can be influenced significantly by hormonal alterations and yet it is able to grow progressively in hypophysectomized hosts. Furth (*24*) has referred to such tumors as being "autonomous, but responsive," which would appear to be somewhat self-contradictory. We would prefer to consider such tumors as being responsive, therefore non-autonomous, and yet not dependent upon known hormonal stimuli for continued growth.

It would appear from a comparison of the results recorded here with

those reported in the literature that mouse-mammary carcinomas must vary not only quantitatively but also qualitatively in their hormone responsiveness. For example, the growth rate of the tumors studied by Mühlbock (*6*) was not influenced by ovariectomy of the host, but in the present studies this procedure increased the growth rate demonstrably. Nevertheless in both studies, the administration of estrogen, under certain circumstances at least, resulted in a slowing of tumor growth. Also in the studies of Liebelt (*10*), both transplanted and spontaneous mammary carcinomas grew more slowly in lactating than in nonlactating animals, while lactation *per se* seemed to be without effect on the growth of the tumor we have studied even though the administration of prolactin increased its rate of growth in ovariectomized females ingesting stilbestrol. What such a spectrum of responses implies relative to the fundamental nature of tumor responsiveness and possibly of tumor dependency certainly remains to be clarified. The work of Foulds (*25*) may give some insight into the mechanism of development of these differing characteristics of responsive tumors. One of the principles which he has proposed and which was derived from his own observations of spontaneous mammary tumors in mice is that the several characteristics which determine the structure and behavior of such tumors may change qualitatively and irreversibly (progression), and that the changes in the different characteristics are independent of each other. This leads to his more general proposition that the structure and behavior of tumors are determined by numerous "unit characters" which within wide limits are independently variable and capable of independent progression and which can be assorted and combined in various ways. Essentially similar conclusions were reached in this laboratory from a study of the characteristics of dependency of a number of testicular interstitial cell tumors of mice (*23*).

Even though the DRV tumor used in these studies had retained a distinctly glandular morphological pattern throughout many transfer generations, its growth response to hormonal alterations was distinctly different from that of normal mammary-gland epithelium. In two situations, castration and estrogen administration, the growth response of the tumor was in the opposite direction from that of the normal epithelium. Similar disparities between the response of neoplastic cells and the cells of the normal tissue from which they arose have been reported in patients with breast cancer being treated with estrogens (*26*), in rats bearing a transplantable mammary fibroadenoma during androgen therapy (*27*), and in mice with transplanted testicular interstitial cell tumors after treatment with a pituitary LH preparation (*23*). Such observations are difficult to reconcile with present-day theories of tumor dependency and responsiveness. During the process of neoplastic transformation, in certain instances, the neoplastic cells retain to a greater or lesser degree the abilities of the normal cells of origin to respond to specific hormonal stimuli. Such apparently enigmatic responses as these may, however, become readily explainable as more is learned about the mechanism of action of hormones in both normal and neoplastic tissues.

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PLATE 105

FIGURE 1.—Histological appearance of DRV tumor transplant showing glandular arrangement that has persisted through 105 transfer generations. × 75

FIGURE 2.—An area of interstitial cells in an ovarian transplant from a female mouse ingesting the stilbestrol-containing diet. *Note* paucity of cytoplasm and pachychromatic nuclear pattern. × 450

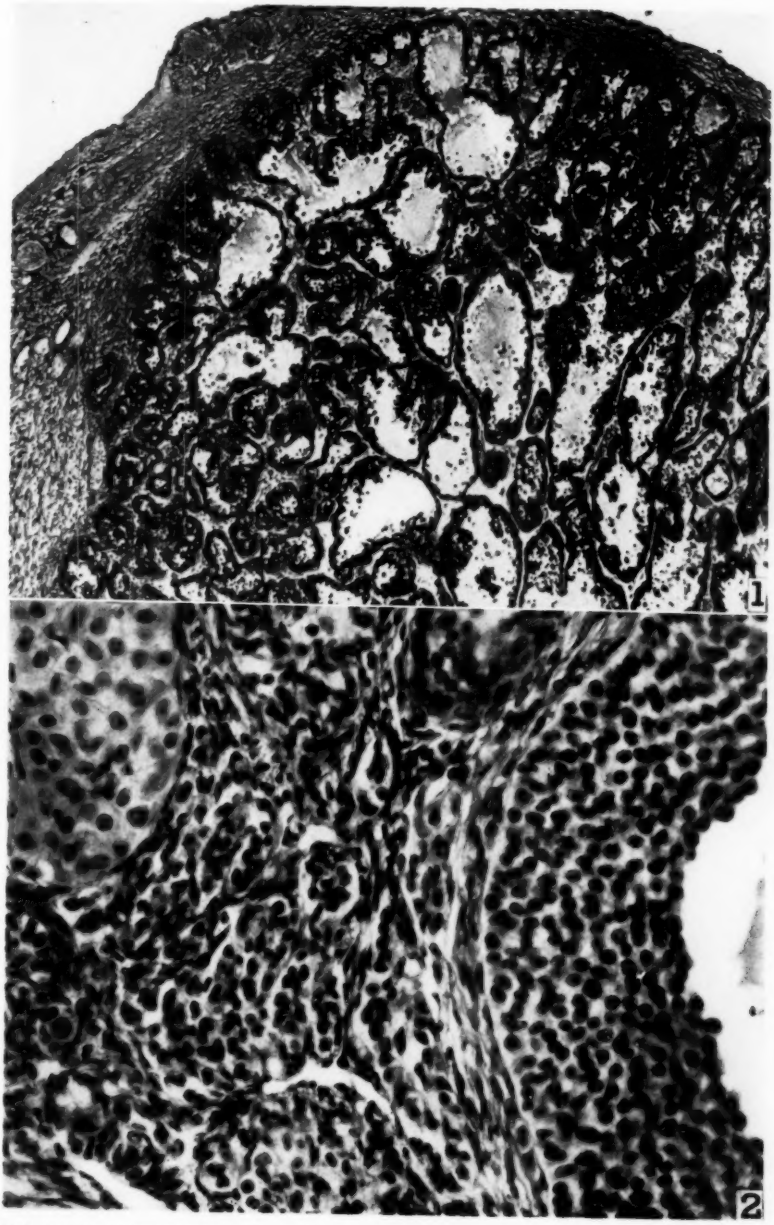

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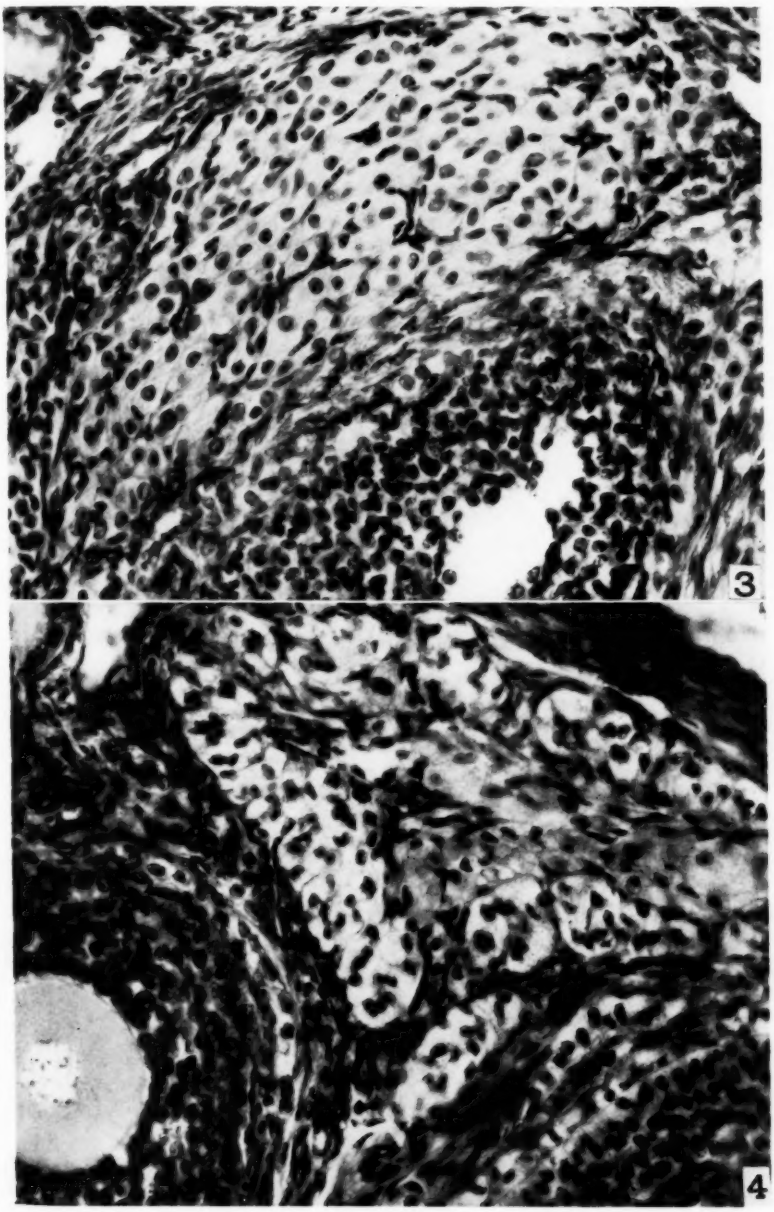
PLATE 106

FIGURE 3.—An area of interstitial cells in an ovarian transplant from a male mouse ingesting the stilbestrol-containing diet. *Note* that cells have much more cytoplasm and their nuclei are not as dense as those in female mice ingesting the same diet. × 450

FIGURE 4.—An area of interstitial cells in an ovarian transplant from a female mouse ingesting the nonstilbestrol-containing diet. × 450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Intracellular and Extracellular Particles in Tissue Cultures Inoculated With Parotid-Tumor Agent (Polyoma Virus) [1]

WILLIAM G. BANFIELD, CLYDE J. DAWE, *and* DARLENE C. BRINDLEY, *Laboratory of Pathology, National Cancer Institute,*[2] *Bethesda, Maryland*

SUMMARY

Cell cultures of murine lymphoma strain P388 D_1 were inoculated with parotid-tumor agent (polyoma virus) and examined with the electron microscope after the appearance of cytopathogenic effect. Intranuclear, intracytoplasmic, and extracellular spherical particles of relatively uniform appearance, measuring approximately 27 to 35 mμ in diameter, were found. Similar particles did not occur in uninfected control cultures. Newborn C3Hf/BiLw mice inoculated with the infected cultures developed salivary-gland tumors and multiple tumors of other types associated with this agent, whereas mice receiving control cultures did not. A second type of particle similar to that described by Dmochowski and Grey in murine and human leukemias was found both in test and control cultures. This type of particle was not associated with leukemia in the present study.—J. Nat. Cancer Inst. 23: 1123–1135, 1959.

THE PROPAGATION of the parotid-tumor agent (polyoma virus) in cell cultures of strain P388 D_1 was first reported by Rabson and Legallais (*1*), using a milk-adapted subline of these cells. Subsequently Dawe, Law, and Dunn (*2*) reported propagation of this agent in the same cell strain grown in a serum-containing medium. A cytopathogenic effect was noted under both these nutritional conditions. More recently, Love and Rabson (*3*) have described in detail the cytochemical changes occurring in milk-adapted P388 D_1 cells infected with parotid-tumor agent (PTA).

The present paper is a report of electron microscopic observations of cultures of P388 D_1 cells infected with PTA, in comparison with similar cell cultures not infected with this agent.

MATERIALS AND METHODS

The isolation of the P388 D_1 cell strain from a transplanted methylcholanthrene-induced lymphoma of a DBA/2 mouse has been briefly

[1] Received for publication May 1, 1959.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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described (*4*), and the derivation of PTA used here has been recorded in detail (*2*). For the present work, the cells were grown in Leighton tubes, in medium containing 40 percent pooled human serum, prepared as described previously (*5*). Cultures were allowed to attain a maximal population of approximately 0.8 to 1.0 $\times$ 10^6 cells before inoculation with the virus preparation. The inoculum for each tube consisted of 0.25 ml. of undiluted supernatant from the preceding culture passage, harvested as soon as the cytopathogenic effect was unmistakably recognizable. At the time of inoculation, each tube also received 0.75 ml. of fresh medium. Control cultures of P388 D_1 cells from the same stock were exposed only to medium from agent-free P388 D_1 cultures. Infected and control cultures were fed identically at 2- to 3-day intervals until the appearance of cytopathogenic effect.

The material examined and described in this report consisted of 4 separate cultures, fixed after the cytopathogenic effect had become evident in varying degree (4 to 11 days following inoculation with virus preparation), and 3 control cultures, fixed at corresponding times. Two of the infected cultures were from the eighth passage of the agent and 2 from the eleventh passage. The supernatants from the eleventh-passage cultures and from the corresponding control cultures were tested for oncogenic potency by injection of 0.05-ml. inoculums of 100:1 dilutions into C3Hf/BiLw mice less than 24 hours old. These animals were from a small breeding colony started with young mice that had given negative complement-fixation and hemagglutination-inhibition tests for infection by PTA.[3]

Eleven of 22 newborn mice inoculated with the diluted material from the infected cultures survived for more than 30 days, and within 3 months all these developed bilateral salivary-gland tumors of the histologic type characteristically associated with PTA. The majority of these mice also bore thymic epithelial tumors, hair-follicle tumors, and small thyroid tumors. Ten of 13 mice inoculated with the control cultures survived more than 30 days, and none developed clinically detectable tumors of any type during an observation period of 4 months.

For the electron-microscope studies, the cells were scraped off the glass and centrifuged in the culture media to form a pellet that was fixed for 3 hours in 1 percent buffered osmium tetroxide according to Palade (*6*). Pieces of 1 mm. or less were cut from the pellet, rinsed briefly in water, then dehydrated in 65, 95, and 2 changes of 100 percent alcohol—½ hour in each. One methyl:7 butyl methacrylate was used for embedding, and polymerization was performed at 60° C.

RESULTS

Particles with an average diameter of 27 to 35 mμ were found in the nucleus, cytoplasm, and on the cell surface in the inoculated cultures,

[3] We are grateful to Dr. W. P. Rowe for the complement-fixation tests.

whereas similar particles were never found in the control cultures. In the crystalline array in figures 3 and 4, they averaged about 35 mμ in diameter, whereas in measurements of individual particles diameters as small as 27 mμ were obtained. The variation in measurement may have depended partly on the plane of section. Their presence in the cells was variable. Often there were only a few cells in a section that contained them. They did not seem to possess a distinct membrane and were of only moderate density. Although in most instances they appeared relatively homogeneous, in some pictures there was a suggestion of inner structure which may become apparent with better resolution.

In some cells (fig. 1), the particles occurred in large numbers throughout the nucleus. Usually, however, they were seen in small, isolated, widely scattered clusters (fig. 2). Infected nuclei often contained irregular clumps of very dense material that sometimes appeared granular (fig. 1). Similar dense clumps have been observed in cells infected with the *Molluscum contagiosum* virus (*7–9*), but it is doubtful that this structure is specific for infection by PTA since a similar change has been observed in a few nuclei (possibly degenerating) of the control cultures.

In some cells (figs. 3, 4, 5, and 6) the particles were present in the cytoplasm and were identical with the nuclear particles. The intracytoplasmic crystalline array shown in figures 3 and 4 was the only one of its kind found. A less compact group of particles was seen in the cytoplasm of an apparently degenerating cell (fig. 5). In other instances the cytoplasmic particles occurred in very loose groups with only a few particles per group (fig. 6, *small arrows*). In figure 6, which shows the clear periphery of a large multinucleate giant cell, there are also particles within two membrane-bound structures (vacuoles). At times they were seen extracellularly and adsorbed to the cell membrane, as illustrated in figure 8.

In addition to the 27- to 35-mμ particles, the eleventh-passage cultures contained intracellular and extracellular particles similar to some of those described by Dmochowski and Grey (*10, 11*) in leukemias of mouse and of man, figure 7. These were about 110 mμ in diameter with a 63- to 85-mμ diameter inner dense body, centrally or eccentrically placed. Within the cell the central body often appeared free, without an outer membrane. Similar particles were present in the control cultures.

DISCUSSION

The 27- to 35-mμ particle described herein was associated only with cell cultures exposed to PTA, and has not been seen in control cultures. That the cultures in which it was seen had tumor-inducing activity was demonstrated by the appearance of parotid-gland and other tumors in mice inoculated at birth with material from the same cultures. Cultures, in which the 27- to 35-mμ particles were not present were inactive.

Kahler and Lloyd (*12*) have made measurements of particles, believed to be PTA, isolated from infected cultures of mouse-embryo mince. The particles measured about 40 mμ in diameter. The 27- to 35-mμ size of the particles reported here is comparable to Kahler and Lloyd's 40-mμ measurement when the effect of the different methods of preparation is considered. Bunting (*13*) found a size difference in the virus of the human skin papilloma. The isolated particles measured 68 mμ, while those in section were only 20 mμ. He attributed the large size of the isolated particles to flattening during drying, and the much smaller size of the sectioned particles to shrinkage during fixation and embedding.

Our observations suggest that the particles multiply within the nucleus. Whether their presence in the cytoplasm is the result of phagocytosis, liberation from the nucleus into the cytoplasm, cytoplasmic multiplication, or all these factors, is not clear. Their presence in vacuoles in the periphery of some cells as illustrated in figure 6 is supportive of phagocytosis as an explanation for their intracytoplasmic location in certain instances.

Particles similar to those associated with murine and human leukemias by Dmochowski and Grey were observed in the eleventh-passage cultures and in the control cultures. However, none of the animals inoculated with this material developed leukemia during the limited period of observation, nor were leukemias observed in any of the several strains of mice receiving inoculums from the first to the tenth passages of the P388 D_1-propagated material (*2*).

Further work is in progress to test the possibility that one or more viruses may be carried concomitantly with PTA in culture, and a search is being made for particles of the 27- to 35-mμ type in tumors induced by the culture-propagated agent.

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PLATES

PLATE 107

FIGURE 1.—Portion of nucleus from infected culture with much of nucleoplasm filled with particles. Nuclear membrane is seen at *upper* and *lower left*. Vacuole in *upper center* represents invagination of nuclear membrane. *Arrows* point to very dense material commonly seen in nuclei of infected cultures. Other material in nucleus probably represents chromatin. × 22,000

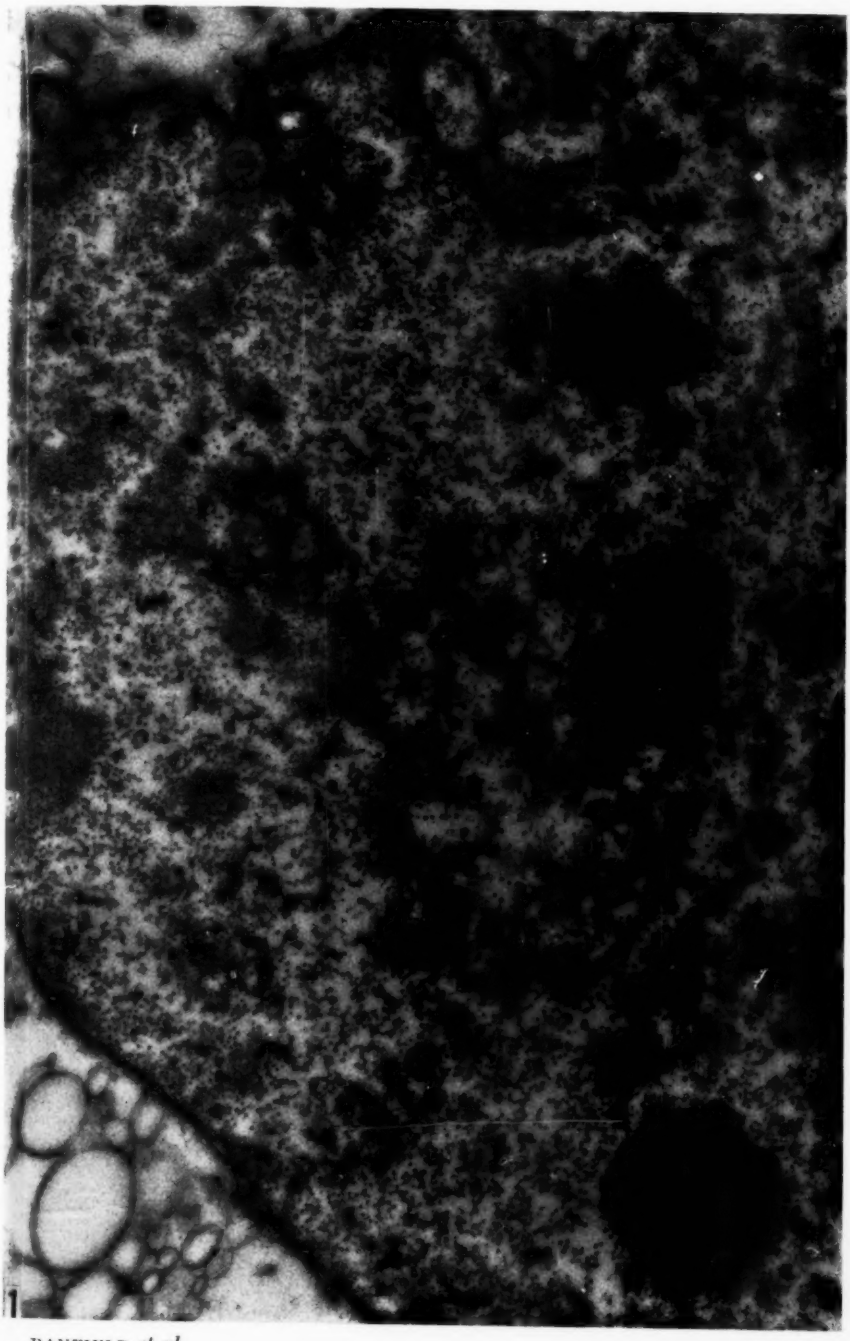

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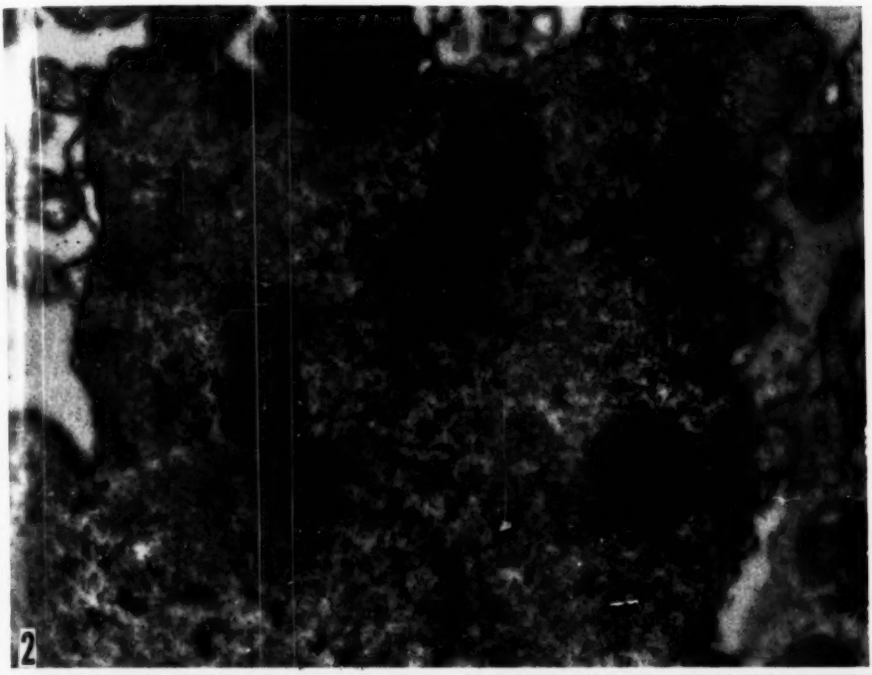
PLATE 108

FIGURE 2.—Portion of nucleus from infected culture. Particles occur in isolated clusters. *Arrow* points to one of the clusters. × 19,000

FIGURE 3.—Crystalline array of particles in cytoplasm. × 35,000

FIGURE 4.—Same as figure 3. × 87,000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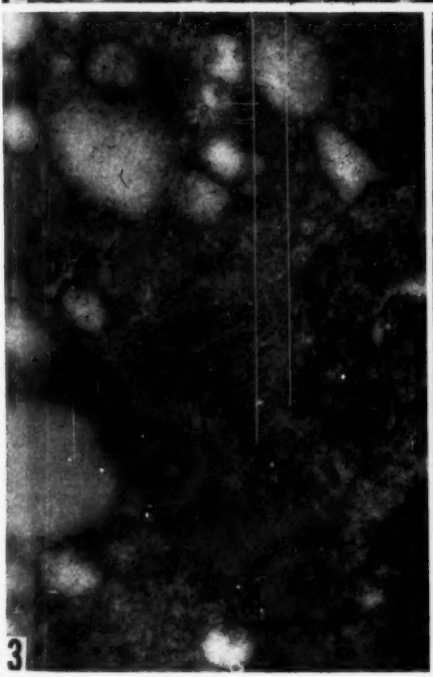
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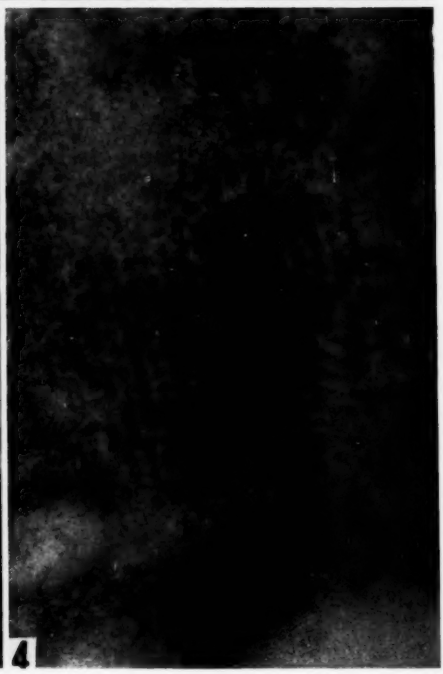
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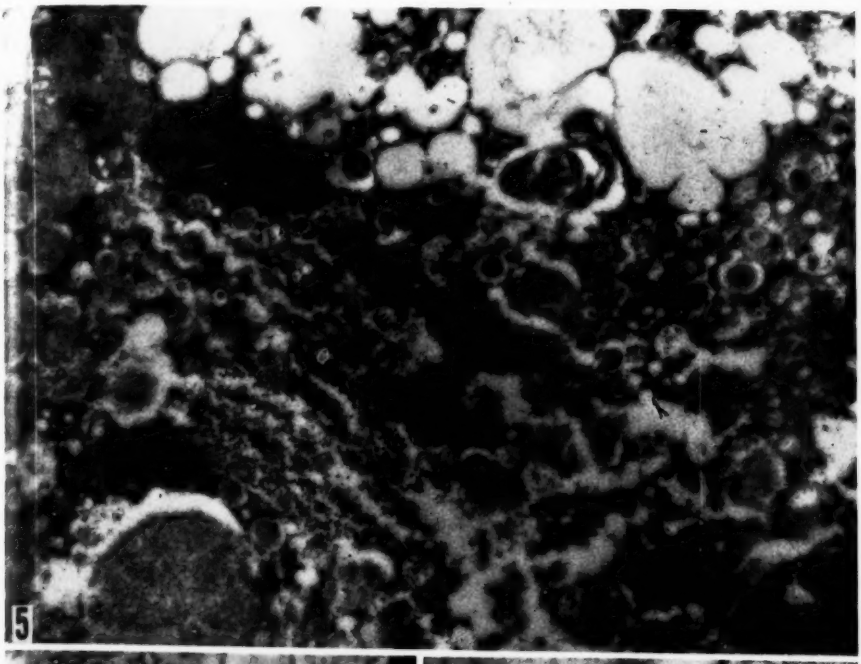
PLATE 109

FIGURE 5.—Relatively compact cluster of particles in cytoplasm of degenerating cell. × 21,000

FIGURE 6.—Clear portion of giant cell containing vacuole-like structures (*large arrows*) filled with particles. They are also free in the cytoplasm (*small arrows*). Portion of central cytoplasm filled with large granules at *lower left*. × 10,500

FIGURE 7.—Larger particles similar to those described by Dmochowski (*10*) in murine and human leukemias. × 21,800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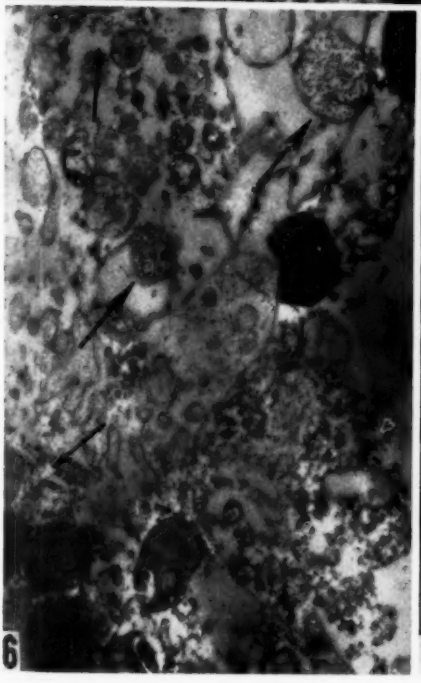
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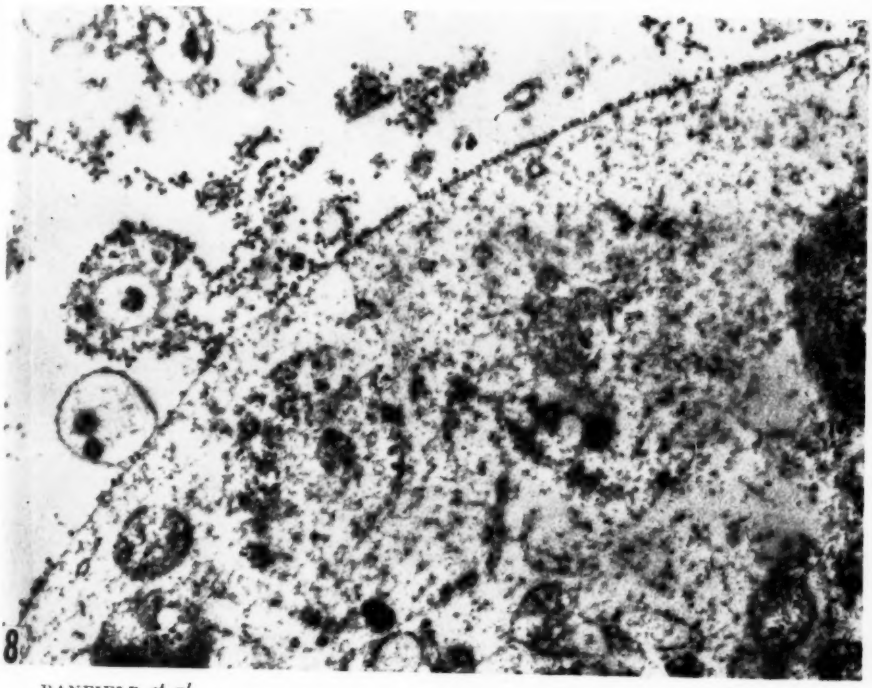
PLATE 110

FIGURE 8.—Portion of cell with many 27- to 35-mμ particles arranged along the cell membrane. Some appear to be free in the extracellular space. × 24,000



Survival of Breast-Cancer Patients in Connecticut, 1935–54 [1, 2]

SIDNEY J. CUTLER, FRED EDERER, MATTHEW H. GRISWOLD, *and* RICHARD A. GREENBERG, *National Cancer Institute,*[3] *Bethesda, Maryland, and Division of Cancer and Other Chronic Diseases, Connecticut State Department of Health, Hartford, Connecticut*

SUMMARY

We are presenting a detailed 20-year survival analysis by stage, age, calendar period of diagnosis, and treatment for cases of breast cancer diagnosed in the State of Connecticut between 1935 and 1954, and are including a review of statistical methodology in survival analysis. Observed survival is measured relative to expected survival in the general population. The 5-year relative survival rate for cases diagnosed during the first 10-year calendar period, 1935–44, was 50 percent. Mortality among the 5-year survivors was far in excess of that expected, based on general population experience. The 5-year relative survival rate for cases diagnosed during the second 10-year calendar period, 1945–54, was 56 percent. There was, however, no evidence of improvement in the 10-year survival rate. In general, there appeared to be little variation in the age-specific survival rates, except that among localized cases, women 75 to 84 years of age had a somewhat more favorable experience than women under 75, and among cases with regional spread, women under 35 had a lower survival rate than older women. The survival results in treatment of breast cancer in Connecticut are similar to those obtained in several well-known treatment centers.—J. Nat. Cancer Inst: 23. 1137–1156, 1959.

THIS IS the first in a series of reports on the survival experience of cancer patients in the State of Connecticut during the period 1935–54. This paper explains the analytical methods used and presents the findings on cancer of the female breast. Reports on patients with cancer of the uterus and of the ovary will follow shortly. These will be followed, in the near future, by reports on cancer of the digestive system and of the skin.

This series, which may well be the most comprehensive study of survival ever attempted in a large population of patients, involved the computation of thousands of rates, ratios, and standard errors. It was made

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[2] This study was made possible, in part, by financial assistance from the National Cancer Institute, Public Health Service. The gathering of the basic data was partially supported by grant 9437; machine processing was supported by contract SA-43-ph-1903.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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possible by the availability of an electronic computer at the National Institutes of Health, United States Public Health Service. Computations which would have required years of work with desk calculators were reduced to hours by the electronic computer. We are indebted to Mr. Elliot Cramer, who ably programed and executed the computations.

The data available from the Connecticut Tumor Registry are unique in that the information pertains to all known cases of cancer in a community rather than a selected series of cases or admissions to a particular institution. (The history and operation of the Connecticut Tumor Registry are described in references *1* and *2*.) Thus it is possible to describe the survival experience of the *average* cancer patient rather than the results obtained in specialized treatment centers or by uniquely qualified medical specialists. Whereas the results obtained under optimum conditions are of interest as a standard of excellence, we are also concerned with the fate of the *average* patient in a *typical* community. It is of interest to compare the *average* with the *best*. Therefore the results reported from several leading medical centers will be compared with the Connecticut findings.

The amount of detail that can be collected effectively in a large-scale cancer registration program must of necessity be limited to items generally available in hospital records and sufficiently simple to make possible uniform reporting. More detailed information is best collected on selected groups of patients in connection with special studies in which specific questions are investigated. The routinely collected data may be used with confidence to describe: (1) the characteristics of cancer patients with respect to sex, age, and other demographic characteristics; (2) the nature of the tumor with respect to primary site, stage, and broad histological-type groups; (3) the type of treatment received, classified into broad categories, such as surgery, radiation, surgery plus radiation; and (4) survival time and cause of death, as recorded on the death certificate. The data can also be used to describe changes in the foregoing variables over time. However, data routinely collected in a large-scale registration program must be used with caution in a comparison of results obtained with various methods of treatment. Registry data generally provide no information on the reasons for the selection of particular therapeutic approaches for individual patients. Patients in the various treatment categories may differ with respect to the presence of complicating nonmalignant conditions, but this possibility cannot be evaluated from the information usually available in a cancer registry.

In spite of these limitations, registry data may provide useful clues on the relative efficacy of different methods of treatment. Careful analysis of temporal changes in survival results, changes in treatment policy, and differences in survival results obtained with different treatment methods can lead to the identification of specific issues for further study. This principle will be demonstrated with actual data, in this series of papers.

METHODOLOGIC REVIEW

In an earlier communication (*3*), an arithmetic index called the *annual survival ratio* was presented as a measure for evaluating the survival experience of cancer patients in terms of the survival experience of the general population. The use of general population survival experience as a frame of reference for evaluating the efficacy of cancer therapy was suggested by Greenwood in 1926 (*4*). In discussing an objective definition of success in the treatment of cancer, Greenwood stated: "If an examination of the after histories of such patients showed that on the average they lived as long as a random sample of persons of the same age, sex, occupation, etc., nobody could object to the use of the word 'cure.' " He suggested, "that a numerical measure of cure must take into account of three quantities, (1) the average duration of life of a person, of the same age as the patient; (2) the average duration of life of a person suffering from the disease and left untreated; (3) the average duration of life of a person suffering from the disease and treated in the manner the value of which it is desired to test." He presents an illustrative example of the suggested numerical measure, which he interprets as follows: ". . . the untreated patient enjoys 15 percent of the normal lifetime and the treated patient 50 percent of the normal lifetime, hence the treatment confers an average advantage of 35 percent of a normal lifetime and this is what we ought to say, avoiding altogether the ambiguous word 'cure.' " In addition to suggesting a numerical measure for evaluating the total survival experience of a group of cancer patients, Greenwood offered an approach for evaluating the trend of mortality rates observed in the group: "A very interesting question is whether . . . the rate of mortality declines in such a fashion that it might be expected in a reasonable number of years to approximate to a normal figure."

Although Greenwood's proposed index for measuring the "advantage" conferred by therapy is conceptually admirable, it is generally impractical. This is so because the clinical characteristics of untreated cancer patients are rarely comparable to those of treated patients. However, it is possible to compare the survival experience of cancer patients with that of the general population in order to ascertain: (1) the ratio of observed to expected survival, and (2) whether, in time, the mortality rate declines to a "normal" level.

In reporting on the survival experience of patients with breast cancer, Nathanson and Welch (1936) compared the survival curve for the patient population with a "normal" survival curve (*5*). They summarized their findings as follows: "The normal life expectancy, determined from Massachusetts life tables for the age fifty-two, the median of our series, is about twenty years. This is about six times the life expectancy of a woman with a treated carcinoma of the breast of the same age."

Berkson (1942) and Berkson and Gage (1950) presented a method for *correcting* survival rates for deaths not due to cancer that would answer the question: "What is the survival rate so far as cancer is concerned?"

(*6*, *7*). This is accomplished by determining, from general population life tables, the expected survival rate in a population not subject to the risk of dying from cancer. In general practice, the survival rate from all causes of death, including cancer, is used as an estimate of the survival rate in a population subject to the risk of dying from all causes except the specific form of cancer under study. Since each specific form of cancer accounts for a very small proportion of all deaths, no correction of published life-table values is generally required (*8*). This approach avoids the difficulty of establishing the exact cause of death for each patient, since ". . . we are not able to obtain, in many instances in which we know definitely the time of death, any information regarding the cause, and where we do have a report of the cause, it is not generally reliable" (*7*). Paterson, Tod, and Russell (*9*) support this point of view, and state: ". . . no figures which depend on opinion as to the cause of death . . . can be accepted as completely reliable, particularly if they are to be used to compare the value of different methods of treatment." ". . . death from intercurrent disease may be interpreted in two ways: it may mean that at the time when the patient died of another disease or injury no sign of cancer could be found, or it may mean that he died of another cause but with cancer present. Even were agreement on interpretation to be reached, it is still a matter of opinion dependent on skill in examination whether there were signs of cancer or not, and whether the supposed other cause was not itself another manifestation of the disease."

The *corrected rate* (Berkson) is obtained by the formula: $p_c = p/p_0$, in which p is the observed total survival rate in a group of cancer patients, and p_0 is the survival rate in the general population from causes other than cancer. The quantity p_c is interpreted as "the survival rate if cancer alone were the cause of death." In other words, p_c represents the estimated proportion of patients that escaped the force of mortality due to cancer. Since p_c may be computed at any point subsequent to the initiation of follow-up, it provides a measure of the proportion of patients that escaped the force of mortality due to cancer, *up to that point*. It does not represent an estimate of the proportion *cured*, because some escapees may succumb to cancer beyond the point at which p_c was computed. The quantity p_c may also be interpreted as the proportion of expected survivors that did in fact survive.

To depict the course of the survival rates for a group of cancer patients, Berkson (*6*) recommended a graphic presentation in which the cumulative survival rate as of the end of each successive year is plotted, as ordinate, against time, as abscissa. He states, "Since mortality rates are relative, that is, they represent the fraction rather than the number who die per year, it is necessary so to plot the curve that the slopes will be proportional to the relative rates. This is accomplished if the survival rates are plotted on a logarithmic scale; the slopes then give exactly at each time the mortality rate." In discussing a chart containing survival curves for several groups of cancer patients and for "a normal population of the

same age as the average age of the patients who have gastric carcinoma," Berkson states: "We note that for about the first seven years the slope of the curve for the patients with carcinoma who had resection is much greater than that for the normal group, but that after this the curves for the two groups are about parallel. This indicates that after about seven years patients who have survived are dying at about the same rate as the persons in the normal group, and hence have about the same life expectancy." Thus Berkson presented a graphic method for arriving at an answer to the question posed by Greenwood—whether, after a reasonable number of years, the rate of mortality declines to a *normal* level.

The *annual survival ratio* proposed by Cutler *et al.* (*3*) provides a simple arithmetic method for measuring the parallelism between the survival curves for a patient population and a *normal* population, comparable with respect to sex and age. Thus, the trend of mortality rates observed in a population of cancer patients can readily be evaluated in terms of *expected rates* based on general population experience.

SPECIFIC METHODOLOGY

In this study, the actuarial method was used in analyzing survival. An important advantage of the actuarial method for analyzing survival is that it makes possible the use of all survival information accumulated up to the closing date of the study (*10*). In calculating a 5-year survival rate, computations need not be restricted to patients diagnosed more than 5 years prior to the closing date of the study; in calculating a 10-year survival rate, computations need not be restricted to patients diagnosed more than 10 years prior to the closing date of the study. Thus all information accumulated through December 31, 1954, was used in analyzing survival experience among patients during the period 1935 through 1954. Under this procedure, the *effective number* of patients contributing information to the calculated survival rate declines with survival time. The *effective number* is the number of patients in a sample in which all members would be followed to death or to the end of the specified period of observation and which would yield a survival rate and a standard error equal to the values observed. For example, the *effective number* of patients that contributed information to the calculated 15-year survival rate was smaller than the *effective number* that contributed information to the calculated 5-year survival rate. The statistical reliability of the calculated survival rate is, of course, dependent on the *effective number*. This is reflected in the *standard error* of the survival rate, which provides a measure of the extent to which the computed rate may have been influenced by sampling variation. For example, by adding twice the standard error to and subtracting it from the computed survival rate, one obtains an approximate 95-percent confidence interval. This means that in repeated observations under the same conditions the

true survival rate will be within a range of 2 standard errors on either side of the computed rate, an average of 95 times in 100. The standard error of the survival rate has been computed by a method developed by Greenwood. Explanations of its use have been given by Merrell and Shulman (*11*), and by Cutler and Ederer (*10*).

Connecticut life tables for 1940 and 1950 were used in estimating expected survival rates based on general population experience. Values for intermediate years were obtained by straight line interpolation. Connecticut life-table values for 1955 were estimated by applying the observed ratio of Connecticut to United States life-table values for 1950 to the United States life table for 1955. Expected survival rates for 1951 through 1954 were obtained by interpolation.

Two factors were taken into account in calculating the expected survival rates for each follow-up year: (1) the age distribution of the patient population at the beginning of each year, and (2) the secular trend in life-table values for the general population between 1940 and 1955. The procedures generally conform to the recommendations made by Milmore (*8*).

In evaluating patient survival over a period of 20 years, it is important to take into account the changes in the age distribution of the patient population. For example, 19 percent of the breast-cancer patients were between 65 and 74 years of age at the time of diagnosis of disease. At the beginning of the 11th year after diagnosis, the survivors of this segment of the patient population accounted for only 13 percent of all survivors; by the beginning of the 16th year this segment represented only 9 percent of all survivors. To take changes in age distribution into account, the patient population was divided into 7 age groups, as of diagnosis: under 35, 35 to 44, . . . 75 to 84, 85 and over. The midpoint of each age group was used in entering in the life table. In obtaining the expected survival rate for all ages combined, for each follow-up year, the age-specific expected survival rates were weighted by the relative number of survivors among members of each age cohort alive at the beginning of the follow-up year.

The problem of selecting appropriate life tables in estimating expected survival rates was recognized by Stocks (1950), who stated: "It has to be remembered that a life table is based on what would happen if the death rates for the country as a whole in a given year continued to operate without change. With falling death rates the survival actually experienced by a population starting from that year is generally rather better than what was expected according to the life table; and furthermore life tables are not constructed for every year and the latest available may be based on death rates of some years back. Consequently, the comparison is with an expectation not quite so good as the real one at the time the patients were living" (*12*). Stocks did not consider this a serious problem when interest is in obtaining comparative rates, *i.e.*, comparing survival in patient groups whose disease was diagnosed during the same calendar period. However, changes in the general population mortality rates

should be taken into account in comparing patient cohorts whose disease was diagnosed 10 or more years apart. Otherwise, as shown by Milmore (1958): "Among patients with a specified chronic disease, if mortality attributable to that disease has not changed, groups whose disease is of recent onset would nevertheless have survival rates higher than groups whose disease began a decade or more ago, because mortality from other causes has decreased" (*8*).

Changes in the general population mortality rates are also of consequence when one is attempting to determine whether in a reasonable number of years the survival rate for the patient population attains a normal level. For example, if we were to use the 1940 life table in evaluating the long-term survival experience of patients whose disease was diagnosed in 1940, it might appear that approximately 10 years after this diagnosis the survivors were no longer exposed to an excessive mortality risk. However, 10 years after disease diagnosis, these survivors were living in 1950, and should be compared with general mortality experience in 1950, which was at a lower level than in 1940. Thus, failure to take the decline in general mortality rates into account can lead to an erroneous conclusion.

In this analysis, the patient population was divided into 2 cohorts with respect to calendar period of diagnosis: 1935–44 and 1945–54. Each cohort was subdivided into 7 age groups: under 35, 35 to 44, . . . 75 to 84, and 85 and over. In estimating expected survival rates from the Connecticut life tables, all members of a cohort were treated as having had a diagnosis at the midpoint of the calendar period of diagnosis; the midpoint of each age group was used to characterize all its members. For example, for patients whose disease was diagnosed during the period 1935–44, and who were 45 to 54 years of age at that time, the expected survival rates for the 1st follow-up year were based on age 50 in the 1940 life table; the expected rates for the 2d year were based on age 51 in the 1941 life table, the expected rates for the 3d year were based on age 52 in the 1942 life table, etc.

In addition to subdivision by period of diagnosis, age, and primary site, the patient population was further subclassified with respect to extent of disease at diagnosis (stage), and method of treatment during the first course of therapy.

Three classes were used in characterizing the patients with respect to the extent of disease at diagnosis:

1. Localized—cancer confined to the site of origin.
2. Regional spread—cancer had passed the bounds of the site of origin; furthest spread thought to be limited to neighboring organs or tissues, or to regional lymph nodes.
3. Distant or remote spread—cancer had spread to organs or tissues beyond those immediately draining or neighboring the site of origin.

First course of therapy was defined to include all tumor-directed treatment initiated within 6 months of diagnosis. Separate analyses were made for 3 modes of treatment—surgery, radiation, and surgery combined

with radiation. No attempt was made to differentiate between limited and radical surgery; between X ray and other forms of radiation therapy; or between preoperative and postoperative radiation. Such distinctions can be made, if warranted, on selected series of patients, and preferably as part of a special study based on careful review of hospital records by a highly trained medical investigator.

BREAST CANCER

This is a report on 9,934 female patients with primary cancer of the breast diagnosed in Connecticut hospitals during the 20-year period, 1935–54. Virtually all these patients were white. Women with a known prior history of cancer of any site and 83 women with incomplete information were excluded from this study. Ninety-two percent of the diagnoses were microscopically confirmed. The 767 clinically diagnosed cases are included in this analysis for 2 reasons: (1) It is desirable to evaluate the survival experience of all persons in the Connecticut community that were treated for cancer. (2) As shown by Griswold *et al.* (*2*), the survival rates for clinically diagnosed cases are markedly lower than for the microscopically confirmed cases. This suggests that this group is probably made up, in large part, of patients for whom biopsy was considered either medically inadvisable or unnecessary. There seems little reason to question the diagnosis of cancer in the clinically diagnosed cases, though the classification by primary site may be less accurate than by microscopically confirmed cases. In any event, the inclusion of the clinically diagnosed cases tends to depress rather than increase the survival rates. For example, the 5-year survival rate for the total population of breast-cancer cases was 47 percent, in contrast with a rate of 50 percent for the microscopically confirmed cases.

To provide a basis for evaluating changes over time, the patients were divided into 2 groups with respect to period of diagnosis: 1935–44 (3,879 patients) and 1945–54 (6,055 patients). The distribution of cases in each period with respect to age of patient and stage of disease at diagnosis is given in table 1.

Follow-up information accumulated through December 31, 1954, is summarized in table 2. Of the 3,879 cases diagnosed during the first period, 1935–44, 197 were lost to follow-up during the first 10 years after diagnosis; an additional 102 were lost during the next 10 years. Of the 6,055 cases diagnosed during the second period, 326 were lost to follow-up during the 10-year period of observation for this group. The proportion of patients lost to follow-up was relatively small. Thus, the assumption that subsequent to date of last contact the survival experience of lost cases was similar to that of cases remaining under follow-up is not likely to introduce a significant bias. [*See* reference (*10*) or (*11*) for an explanation of the manner in which lost patients and patients "withdrawn alive" are handled in applying the life-table method.]

TABLE 1.—Distribution by age of patient and stage of disease at diagnosis: Female patients with cancer of the breast diagnosed in Connecticut, 1935–54

Period and age at diagnosis	Stage at diagnosis				
	All stages	Localized	Regional	Distant	Stage unspecified
Cases diagnosed 1935–44					
Total	3, 879	1, 508	1, 751	389	231
Under 35	162	68	69	9	16
35–44	710	309	305	51	45
45–54	961	334	482	89	56
55–64	1, 010	363	479	111	57
65–74	732	295	308	88	41
75–84	266	119	100	34	13
85 and over	38	20	8	7	3
Cases diagnosed 1945–54					
Total	6, 055	2, 681	2, 671	404	299
Under 35	261	123	117	11	10
35–44	1, 008	477	446	46	39
45–54	1, 400	585	683	73	59
55–64	1, 446	634	659	99	54
65–74	1, 219	527	526	108	58
75–84	616	288	205	59	64
85 and over	105	47	35	8	15

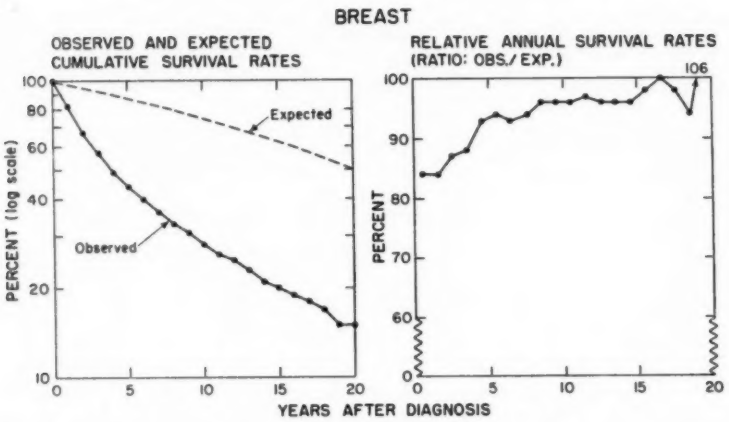
TABLE 2.—Summary follow-up data on female patients with cancer of the breast diagnosed in Connecticut, 1935–54

Period of diagnosis and follow-up interval	Alive at beginning of interval	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval*
Cases diagnosed 1935–44				
0–5 years	3, 879	2, 146	91	0
5–10 "	1, 642	552	106	0
10–15 "	984	201	95	444
15–20 "	244	26	7	211
Cases diagnosed 1945–54				
0–5 years	6, 055	2, 321	262	2, 235
5–10 "	1, 237	261	64	912

*Alive on closing date of study, December 31, 1954.

Twenty Years of Survival Experience

The survival experience, over 20 years of follow-up, of the 3,879 patients with breast cancer diagnosed during the period 1935–44, is depicted in text-figure 1. The logarithmic scale used for plotting the percentage of survivors at the end of successive intervals (left side of chart) permits visual evaluation of the rate of depletion (mortality) during successive annual intervals. A steep slope indicates a high mortality rate; a gradual slope indicates a relatively low mortality rate. The expected survival experience for a group of women from the general population, with an age distribution similar to that of the breast-cancer patients, is shown for comparison. The 2 curves are markedly divergent during the first 10 years. They are less divergent during the next 10 years, but do not



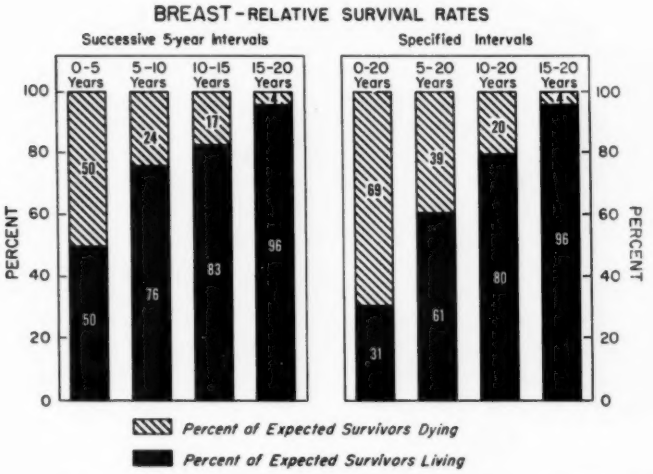
TEXT-FIGURE 1.—Observed and expected survival rates for 20 years of follow-up: Patients with cancer of the breast diagnosed in Connecticut, 1935–44.

become parallel during the 20-year period of observation. This means that the rate of mortality in the population of breast-cancer patients exceeded the rate in the general population throughout the 20 years of follow-up.

To provide a more precise measure of the relationship of the observed and expected survival rates in successive follow-up years, *relative survival rates* were computed. The annual relative survival rate is the ratio of the observed to the expected survival rate for each individual follow-up year. A relative rate of 100 percent means that during a specific follow-up year the mortality rates in the patient and in the general population were equal. A relative rate of less than 100 percent means that mortality in the patient group was greater than expected on the basis of general population experience. The attainment and maintenance of a survival rate in the patient group equal to that in the general population would indicate that excess mortality had been eliminated. Obviously, the basic goal of therapy is to achieve this as quickly as possible for the largest possible proportion of patients.

Relative survival rates for 20 individual years of follow-up are depicted on the right-hand side of text-figure 1. We see that during the first 15 years after diagnosis the relative rates increase fairly steadily, but definitely do not reach the 100-percent level. During the fourth 5-year period, the survival experience of the patient group appears to be approximately normal. By that time, only one fifth of the original group was still alive.

The survival experience of the breast-cancer patients relative to expectation based on general population experience is summarized, over various follow-up intervals, in text-figure 2. The relative survival rate for the first 5 years was 50 percent; during the second 5 years, 76 percent (left side of chart). This indicates that one half of the patients expected to survive to the end of the 5th year, in actual fact, died. Of the patients



TEXT-FIGURE 2.—Relative survival rates for specified follow-up intervals: Patients with cancer of the breast diagnosed in Connecticut, 1935–44.

alive at the end of the 5th year, one fourth of the number expected to live to the end of the 10th year, in fact, died. The right-hand side of figure 2 indicates relative survival rates to the end of the 20th year. For example, the relative survival rate from the end of the 10th to the end of the 20th year was 80 percent; one fifth of the expected survivors died. Thus we see that breast-cancer patients alive 10 years after diagnosis of cancer were not free of an excessive mortality risk.

Survival rates for patients with breast tumors that were classified as *localized* and for patients with tumors classified as *regional* are given in table 3. We see that even among those with *localized tumors*, the relative survival rates fail to achieve a normal level. The relative rates in the 4 successive 5-year intervals after diagnosis were 76, 83, 88, and 93 percent. Of the patients with *localized tumors* alive at the end of the 10th year, only 82 percent of the expected number of survivors were actually alive at the end of the 20th year, which is a very disturbing finding. Much more detail than is here available is needed to evaluate the observed continuing excessive mortality among breast-cancer patients. As a first step, we are now compiling information on the distribution of causes of death with respect to survival time. Is the excessive mortality 10 and more years after diagnosis of disease attributable to cancer or to other disease categories?

Secular Trend

The 5-year relative survival rate for patients with breast cancer diagnosed more recently (1945–54) was somewhat higher: 56 percent compared with 50 percent for patients with cancer diagnosed between 1935 and 1944 (text-fig. 3). Part of this difference resulted from an increase

TABLE 3.—Twenty years of survival experience, by stage of disease: Female patients with cancer of the breast diagnosed in Connecticut, 1935–44*

Cumulative survival rates from date of diagnosis

Interval in years	All stages†		Localized		Regional	
	Crude	Relative	Crude	Relative	Crude	Relative
0– 5	44	50	65	76	35	40
0–10	28	38	46	63	21	27
0–15	20	32	34	55	13	21
0–20	15	31	25	51	12	22

Relative survival rates for 5-year intervals

Interval in years	All stages†		Localized		Regional	
	Rate	Standard error	Rate	Standard error	Rate	Standard error
0– 5	50	0. 9	76	1. 4	40	1. 2
5–10	76	1. 4	83	1. 8	69	2. 3
10–15	83	2. 3	88	2. 8	76	4. 2
15–20	*96*	5. 7	*93*	7. 8	*106*	5. 1

Relative survival rates to end of 20th year

Interval in years	All stages†		Localized		Regional	
	Rate	Standard error	Rate	Standard error	Rate	Standard error
0–20	31	2. 2	51	4. 7	-22	2. 0
5–20	61	4. 3	*68*	6. 1	56	4. 7
10–20	*80*	5. 4	*82*	7. 1	*81*	6. 3
15–20	*96*	5. 7	*93*	7. 8	*106*	5. 1

*All survival rates are expressed as percentages. Relative survival rates are the ratio of observed to expected survival. Rates in italics have standard errors between 5.0 and 9.9.

†Includes cases classified as "distant" and "stage unspecified." Of the 389 patients with breast cancer diagnosed after distant metastases had been manifested, only 29 percent survived one year; 2 percent survived 5 years. Of the 231 patients classified as "stage unspecified," 39 percent survived 5 years; 20 percent survived 20 years.

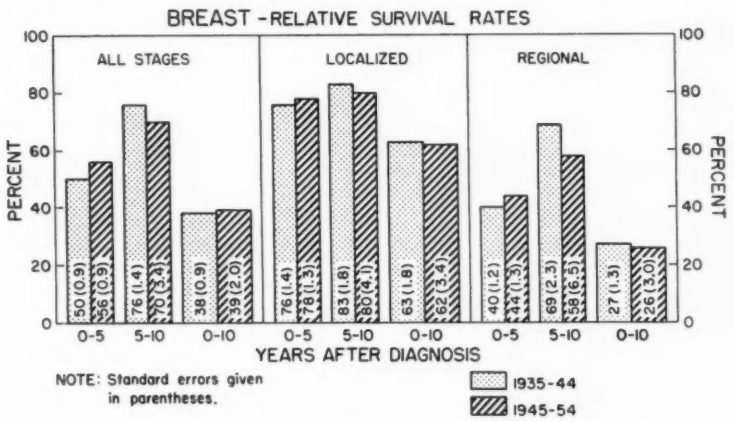
in the percentage of breast cancers classified as localized: 44 percent during 1945–54 compared with 39 percent during 1935–44 (table 1). There was no significant improvement in the 5-year survival rate among patients with localized cases (an increase from 76 to 78 percent). The increase was greater among patients with regional spread of the neoplasm (from 40 to 44 percent).

Examination of the survival experience from the end of the 5th to the end of the 10th year after cancer diagnosis reveals a very surprising phenomenon. During the first 5 years after diagnosis the survival rates were a little better in the later cohort, but the reverse was true during the second 5 years. The observed reversal is statistically not very reliable. The standard errors for the rates from the end of the 5th to the end of the 10th years are large. It is therefore premature to speculate about possible causes. However, whatever the significance of this observed reversal, there is no evidence of improvement in the 10-year relative survival rate. This is discouraging in view of the reported increase in the percentage of cases classified as localized at diagnosis.

Variation by Treatment

Treatment received by breast-cancer patients, during the first course of medical care, was classified into 3 broad categories: surgery, radiation, and surgery plus radiation. The data at hand do not provide information on extent of surgery, type of radiation, radiation dosage, and whether radiation was administered preoperatively or postoperatively.

Approximately 9 out of every 10 breast-cancer patients were treated surgically during each period. However, surgery in combination with radiation was used less often during the latter period; the percentage of patients treated by combination therapy decreased from 27 to 18 percent. This change occurred among patients with regional spread at diagnosis, as well as among those with localized cases (table 4).



TEXT-FIGURE 3.—Comparison of relative survival rates for patients with breast cancer diagnosed during 1935–44 and 1945–54: Patients with cancer of the breast diagnosed in Connecticut.

Relative survival rates with respect to treatment during the first course of medical care are given in table 5. These data are presented to describe the survival pattern within each treatment group rather than to evaluate the relative merits of different methods.

As might be expected the most favorable survival experience was observed among patients with localized tumors treated by surgery only. This group is probably the one with the best prognosis on the basis of the characteristics of the tumor and the general health of the patient. It is therefore noteworthy that mortality in this group was excessive throughout the first 15 years after diagnosis. The annual relative survival rates did not reach the 100-percent level until the 16th year.

Breast-cancer patients treated by radiation only accounted for a very small proportion of all cases (table 4) and undoubtedly represent a highly selected group made up for the most part of poor operative risks. The low survival rates for this group are therefore not surprising.

TABLE 4.—Percentage distribution by treatment during first course of therapy according to stage of disease and period of diagnosis: Female patients with cancer of the breast diagnosed in Connecticut, 1935–54

Type of treatment	Stage at diagnosis							
	All stages*		Localized		Regional		Distant	
	1935–44	1945–54	1935–44	1945–54	1935–44	1945–54	1935–44	1945–54
Total number of cases	3,879	6,055	1,508	2,681	1,751	2,671	389	404
Percentage distribution								
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Surgery	61.3	73.4	75.1	86.3	58.2	68.8	23.9	26.0
Radiation	6.8	2.6	2.5	0.8	5.0	1.8	31.4	17.1
Surgery plus radiation	26.5	17.5	19.9	10.6	34.5	26.1	14.9	10.9
Other treatments	0.0	2.3	0.0	0.6	0.0	1.5	0.0	17.3
No treatment	5.4	4.2	2.5	1.7	2.3	1.8	29.8	28.7

*Includes "stage unspecified."

TABLE 5.—Relative survival rates by treatment during first course of medical care and stage of disease: Female patients with cancer of the breast diagnosed in Connecticut, 1935–44 and 1945–54*

Treatment and years after diagnosis	All stages†		Localized		Regional	
	Relative survival rate	Standard error	Relative survival rate	Standard error	Relative survival rate	Standard error
Cases diagnosed 1935–44						
Surgery						
0–5	59	1.2	80	1.6	42	1.7
5–10	79	1.7	87	2.0	68	3.1
10–15	83	2.8	89	3.2	*73*	6.0
15–20	*105*	6.4	*105*	8.6	*111*	5.7
Radiation						
0–5	11	2.2	*33*	9.0	12	4.2
Surgery plus radiation						
0–5	48	1.7	72	3.0	40	2.2
5–10	70	2.6	74	3.8	70	3.8
10–15	83	4.1	*84*	6.0	*81*	6.2
Cases diagnosed 1945–54						
Surgery						
0–5	63	1.0	81	1.4	46	1.6
5–10	69	4.2	82	4.6	*50*	9.6
Radiation						
0–5	6	2.7	—	—	*6*	5.6
Surgery plus radiation						
0–5	50	2.1	69	3.6	46	2.6
5–10	*72*	5.7	*77*	9.6	*72*	6.1

*All survival rates are expressed as percentages. To obtain the 10-year survival rate multiply the rate for the first 5 years by the rate for the second 5 years. Multiply this result by the rate for the third 5 years to obtain the 15-year survival rate etc., Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors equal to 10.0 or larger are not shown.

†Includes "distant" and "stage unspecified."

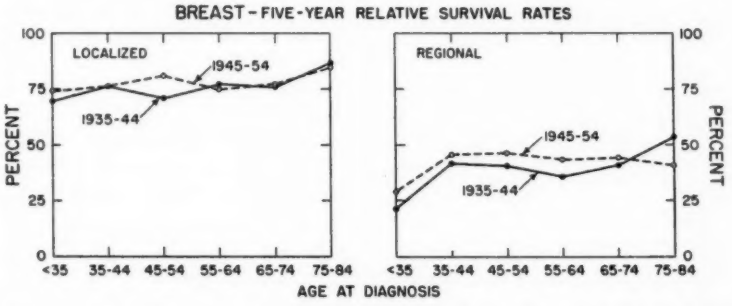
Variation by Age

Nathanson and Welch (*5*) investigated the relationship between age at diagnosis of cancer and survival and found that cancer of the breast appeared to be more malignant in the young than in the old. They reported that, "The median length of life in the age group below forty is about three years, from forty to sixty about three and a half years, and about four years thereafter." Since younger women would normally be expected to have a longer life expectancy than the older ones, the absolute differences in median survival time reported by Nathanson and Welch understate the relative excess in mortality among young breast-cancer patients.

In assessing the relationship between survival and age from the Connecticut data, normal survival expectancy was taken into account. Relative 5-year survival rates are given for each of 6 age groups, *i.e.*, the observed survival rate is expressed as a percentage of the expected survival rate. This procedure takes account of differences in the total mortality risk among members of each of the 6 age groups. Thus the relative survival rates reflect the results of the force of mortality due to cancer of the breast, as experienced by members of each age group.

The Connecticut data indicate that when breast-cancer patients are considered as one group, irrespective of stage of disease or method of treatment, the relative survival among patients under 35 years of age was a little lower than the average for all ages, whereas relative survival among patients 75 through 84 years of age was a little higher (table 6). (Relative survival rates were not computed for women 85 years and over because of the small number of cases and the unreliability of estimated survival rates for this group.) The differences were not marked during either calendar period of diagnosis (1935–44, 1945–54). There was little variation in the relative survival rates for the 4 other age groups.

When the age-specific relative survival rates are examined separately for localized and regional cases, an interesting contrast shows up (text-fig. 4). Among localized cases, there is little variation in the rates for the



TEXT-FIGURE 4.—Five-year relative survival rates by stage and age at diagnosis: Patients with cancer of the breast diagnosed in Connecticut during 1935–44 and 1945–54.

TABLE 6.—Relative 5-year survival rates by age at diagnosis of cancer, according to stage of disease and treatment during first course of medical care: Female patients with cancer of the breast diagnosed in Connecticut, 1935–44 and 1945–54*

Stage and treatment	Calendar period	Under 35		35–44		45–54		55–64		65–74		75–84	
		Relative survival rate	S.E.†	Relative survival rate	S.E.	Relative survival rate	S.E.	Relative survival rate	S.E.	Relative survival rate	S.E.	Relative survival rate	S.E.
All stages‡													
	1935–44	40	3.9	54	1.9	48	1.7	47	1.7	52	2.3	*63*	5.4
	1945–54	51	4.0	57	2.0	58	1.7	54	1.7	53	2.2	59	4.2
Localized‡													
	1935–44	*69*	5.9	76	2.6	71	2.7	77	2.7	76	3.8	*87*	8.4
	1945–54	*74*	5.1	76	2.6	81	2.3	75	2.5	77	3.3	*85*	6.3
Surgery only													
	1935–44	*70*	6.9	80	2.8	74	3.0	81	3.0	77	4.2	*95*	9.8
	1945–54	*80*	5.0	77	2.9	83	2.4	77	2.6	81	3.6	*91*	6.8
Surgery plus radiation													
	1935–44	n.r.		*66*	5.6	*65*	5.8	*74*	6.0	*94*	8.8	n.r.	
	1945–54	n.r.		*74*	6.7	*74*	6.7	*68*	6.7	*69*	9.9	n.r.	
Regional involvement‡													
	1935–44	*22*	5.0	42	2.9	41	2.3	36	2.3	41	3.5	*53*	8.6
	1945–54	*29*	5.8	46	3.0	47	2.4	43	2.6	44	3.2	*41*	6.6
Surgery only													
	1935–44	*22*	8.7	40	4.2	42	3.0	41	3.0	44	4.6	n.r.	
	1945–54	*34*	8.4	46	3.7	47	3.0	43	3.0	47	3.7	*47*	8.2
Surgery plus radiation													
	1935–44	*22*	6.6	46	4.2	44	4.0	29	4.0	*46*	7.2	n.r.	
	1945–54	*32*	9.4	*47*	5.4	51	4.2	*45*	5.1	*44*	8.0	n.r.	

*All survival rates are expressed as percentages. Rates in italics have standard errors between 5.0 and 9.9. Rates with standard errors equal to 10.0 or larger are not shown (n.r.).
†S.E. = standard error.
‡All methods for treated patients and those untreated combined.

5 age groups under 75, but the survival experience among women 75 to 84 years of age was more favorable. This relationship shows up more clearly among patients treated by means of surgery only than in patients treated by surgery in combination with radiation. In contrast, among regional cases, relative survival among women under 35 years was low, and the rates for the 5 other age groups were fairly close. This was true for patients treated by either treatment regimen, surgery only, and surgery combined with radiation.

Comparison With Other Series

Comparison of results reported by different investigators is generally difficult, because one is never sure that the patients in the various series are truly comparable. Superior results achieved in one treatment center as contrasted with another may be due to differences in the characteristics of the 2 patient groups with respect to age, race, socioeconomic status, general clinical condition, and the extent of the cancer at diagnosis. Insufficient detail is usually reported to permit adjustment for differences with respect to all these factors. The results are frequently not reported uniformly and there may be differences in the definition of terms. In addition, one series may include all diagnosed cases, both treated and untreated, and another series may be restricted to cases treated in a specified manner. It is regrettable that investigators so often fail to define the series they are reporting, and neglect to report the number and type of cases excluded. Although, for example, exclusion of operative or intercurrent deaths precludes the evaluation of total experience, it would be extremely helpful to know (a) that these exclusions were made, and (b) how many.

Despite these limitations, comparisons are interesting and are constantly attempted. At this time, it is of interest to compare the results obtained in the State of Connecticut with results reported from some leading treatment centers, to ascertain whether the results obtained under *average* conditions are markedly different from results obtained under *optimum* conditions.

In discussing the comparison of the results obtained at the Mayo Clinic with those reported by McWhirter, Berkson *et al.* (*13*) state: "We believe this comparison, using only the patients coming under treatment, is the most meaningful one; but in present circumstances we think it to the point to make the comparison including literally all patients—*i.e.*, including McWhirter's 273 patients (15%) who did not come under treatment, and the 362 patients (16%) in the Rochester series found inoperable, that is, patients who did not have a radical mastectomy because of the advanced character of the cancer." Berkson then computes 5-year survival rates for 1,882 patients seen in Edinburgh and 2,262 patients seen at the Mayo Clinic during 1941–47. The 5-year survival rate for the Edinburgh patients was 42 percent; the corresponding rate for those in the Mayo Clinic was 50 percent. Thus 50 percent represents the 5-year

survival rate for the total population of breast-cancer patients seen at the Mayo Clinic, 1941 through 1947. This result may be compared with the total experience in Connecticut. For the patients diagnosed during 1935–44, the crude 5-year survival rate (unadjusted for normal life expectancy) was 44 percent. The corresponding rate for the patients diagnosed 1945–54 was 49 percent. In an earlier report, Griswold (*2*) indicated that for patients with breast cancer diagnosed in 1941 through 1946, the 5-year survival rate was 47 percent. Thus it appears that the survival results for all breast-cancer patients in Connecticut are not different from the results at the Mayo Clinic.

Whether the patients seen at the Mayo Clinic were comparable to the patients in Connecticut with respect to all relevant characteristics is not known. Of the total group in the Mayo series, 16 percent "did not have a radical mastectomy because of the advanced character of the cancer" (*13*). Of the 9,934 patients in the Connecticut series, 42 percent of the cases were classified as *localized*, 44 percent as *regional*, 8 percent as *distant*, and 6 percent as *stage unspecified*. Presumably all of those with *localized* cancer and none with *distant* cases would be considered eligible for radical mastectomy at Mayo. Whether all of those with *regional* cases and some of those with *unspecified* cases would be considered eligible is not known. However, the distribution of patients with respect to eligibility for radical mastectomy is roughly similar in the 2 series.

It is unfortunate that truly comparable data for various subgroups of breast-cancer patients treated at the Mayo Clinic and in Connecticut are not available. It would, for example, be desirable to compare the survival rates for all those with *localized* cases, irrespective of treatment method. We can, however, from the data in table 7, compare survival rates for patients treated by means of radical mastectomy at the Mayo Clinic with all surgically treated patients (limited and radical cases) in Connecticut. We note that the 5-year survival rates at the Mayo Clinic exceed those in Connecticut by a substantial margin. This difference may be due to (1) a superiority of radical surgery over limited surgery, (2) better quality of treatment at the Mayo Clinic, or (3) a more highly selected group eligible for radical surgery at the Mayo Clinic. Whatever the difference between these subgroups may be attributable to, it is of interest to note that when all patients seen at the Mayo Clinic are compared with all patients seen in Connecticut (table 7), there is little difference in the observed survival rates.

Shimkin *et al.* (*15*) reported on the survival of breast-cancer patients seen at the University of California Hospital. For 430 women treated by radical mastectomy, with or without radiation, during 1930–44, the 5-year survival rate was 52 percent. In Connecticut, for cases diagnosed during 1935–44, the 5-year survival rate for patients treated by surgery, both radical and limited, with or without radiation, was 49 percent.

Lewison (*16*) reported on breast-cancer patients seen at the Johns Hopkins Hospital in 1941–45. For patients with localized disease the 5-year survival rate was 69 percent; the 10-year rate was 49 percent. In

TABLE 7.—Comparison of crude 5-year survival rates for cancer of the breast in Connecticut and the Mayo Clinic—survival rates are expressed as percentages

Stage	Mayo Clinic			Connecticut		
	Calendar period	Number of cases	Crude 5-year survival rate	Calendar period	Number of cases	Crude 5-year survival rate
	All diagnosed cases*			All diagnosed cases†		
All stages	1941–47	2, 262	50	1941–46	2, 760	47
	Radical surgery‡			Limited and radical surgery		
All stages	1935–44	2, 480	59	1935–44	3, 456	49
	1945–54	2, 774	63	1945–54	5, 556	53
Localized	1935–44	1, 067	80	1935–44	1, 455	68
	1945–54	1, 405	80	1945–54	2, 634	70
Regional	1935–44	1, 413	42	1935–44	1, 642	37
	1945–54	1, 369	46	1945–54	2, 551	41

*Source: reference (*13*). †Source: reference (*2*). ‡Source: reference (*14*).

Connecticut, for all *localized* cases diagnosed in 1935–44, the 5-year rate was 65 percent, and the 10-year rate was 46 percent. Among patients with regional spread of the disease, the 5-year survival rate at Johns Hopkins was 30 percent; in Connecticut, 35 percent; the 10-year rate at Johns Hopkins was 16 percent; in Connecticut, 21 percent.

Admittedly, the figures on which the foregoing comparisons were made are crude. It seems unlikely, however, that refinement of the data, to adjust for differences among the series with respect to patient characteristics, would materially alter the picture. The results in treatment of breast cancer obtained in 36 hospitals in Connecticut are similar to those obtained in several well-known treatment centers.

Current methods for treating breast cancer have been in use for more than half a century. The Halsted technique, or some modification of it, has been generally accepted as the treatment of choice. The similarity of the observed survival results suggests that variations in technique among the various centers do not materially affect the survival results. The data also suggest that the quality of surgical practice in the treatment of breast cancer in Connecticut is comparable to that in several leading medical centers.

Lewison (*16*) has observed that, "Despite the tremendous strides of our healing art, breast cancer mortality has not yet reflected to any major degree the abounding benefits of an ever-improving surgical skill. It is indeed a most curious and disconcerting fact that the over-all standardized breast cancer death rate for females in the United States has remained virtually unchanged during the past quarter of a century" (1930–55). There are no national figures available on the incidence of breast cancer, but the Connecticut registry data indicate that the incidence of breast cancer has been stable from 1935 through 1954 (*17*). A stable incidence rate and a stable mortality rate imply a stable survival rate. This is confirmed by the data presented in this report (text-fig. 3). The

10-year survival rate for cases diagnosed during 1935–44 was 38 percent. The corresponding rate for cases diagnosed during 1945–54 was 39 percent.

The stability of survival rates for breast cancer should not be interpreted as an indication that treatment is futile. However, from the fact that incidence, mortality, and survival rates have all been stable for a good many years, we infer that future improvement is more likely to result from the development of new therapeutic techniques rather than from further refinement of current methods.

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Morphologic Changes in Salivary-Gland Tissue of the Newborn Mouse Exposed to Parotid-Tumor Agent *In Vitro* [1, 2]

CLYDE J. DAWE *and* LLOYD W. LAW,[3] *Laboratory of Pathology and Laboratory of Biology, National Cancer Institute,*[4] *Bethesda, Maryland*

SUMMARY

Salivary-gland tissue of newborn C3Hf/BiLw mice was excised and exposed to parotid-tumor agent (polyoma virus) before transferral to tissue cultures. The cultures were examined microscopically at intervals from 5 to 48 days after explantation. Control cultures were prepared and examined in the same way, without exposure to the agent. In both the test and control cultures at 5 and 12 days there was atrophy and necrosis within the explants with no significant difference between the exposed and unexposed tissues. From the 19th day onward, differences between the exposed and unexposed cultures were consistently evident. Salivary-gland epithelium in the test cultures developed characteristics very similar to those of salivary tumor cells *in vivo*. There was active proliferation with filling of the glandular lumens by altered cells. Solid cords of cells extended from the glands and reproduced the patterns of the analogous tumors *in vivo*. The mitotic index was higher in the test cultures than in the controls. Cytologic changes characteristic of salivary tumors *in vivo* also were noted in the exposed cultures. Nuclei were enlarged, nucleoli more prominent, and intranuclear, clear spaces were often present, sometimes surrounding a large, amphophilic body. The cytolytic effect of the agent was apparent *in vitro* as *in vivo*. It is concluded that the parotid-tumor agent acts on the salivary-gland epithelium, without intermediation of other organs, to produce morphologic changes similar to those seen *in vivo*.—J. Nat. Cancer Inst. 23: 1157–1177, 1959.

AS A step in the investigation of mechanisms of action of the parotid-tumor agent (polyoma virus) first reported by Gross (*1*) and by Stewart (*2*), it is important to determine whether this agent acts directly on the cells of the tissues destined to yield tumors. If the agent does not act directly on the target tissue, an alternative possibility, suggested by the large number of sites at which tumors develop under the influence of the agent, is that some intermediate influence (*e.g.*, endocrine) is first activated by it.

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To probe this question, salivary glands of newborn mice were excised, exposed to parotid-tumor agent (PTA), placed in tissue culture, and observed periodically for cellular changes comparable to those previously reported in salivary glands *in vivo* (*3*).

MATERIALS AND METHODS

Mice.—The salivary glands were obtained from C3Hf/BiLw mice not more than 24 hours old. The parents of all donor animals were kept in an animal room from which all animals experimentally exposed to PTA were excluded. Four of the 5 mothers of donor newborns gave negative tests for complement-fixing (CF) and hemagglutination-inhibiting (HI) antibodies against PTA, after delivery of their offspring.[5] The fifth mother was not tested. Four litters of 6 mice each and one litter of 7 were used. The salivary glands from each litter were divided equally between test and control cultures.

PTA.—The agent used was harvested from its tenth serial passage in continuous culture cell strain P388 D_1 (*4*), isolated from a lymphoma of a DBA/2 mouse. The propagation of this same strain of agent in this cell line has been described by Rabson and Legallais (*5*) and by us (*6*). In the present work, the P388 D_1 cell cultures were carried in serum-containing growth medium of the same composition as that in which they had originally been isolated. The composition of this medium has been described previously (*7*). In the first 3 culture series, to avoid loss of activity, the harvested cultures were not filtered, but cells were broken by freezing and thawing, and the resulting material was centrifuged at 3000 rpm for 10 minutes. The supernatant was dispensed into ampoules and preserved at −20° C. There was no evidence of growth or survival of P388 D_1 cells in any cultures in which this preparation was used. For the fourth culture series, the same preparation of agent was passed through an ultrafine fritted-glass filter. The filtrate was tested for presence of P388 D_1 cells by intraperitoneal injection of 0.10 ml. into each of 3 (BALB/c × DBA/2)F_1 mice. A control preparation was made by freezing and thawing cultures of P388 D_1 cells not infected by PTA, and passing them through an ultrafine fritted-glass filter. This filtrate was tested for P388 D_1 cells in the same manner as the agent-containing preparation. No P388 tumors appeared in any mice receiving these test and control materials.

The tumor-inducing activity of the agent preparation was tested in (C3Hf × AKR)F_1 newborn mice obtained by mating C3Hf/Lw females to AKR/Lw males. Of 29 animals receiving subcutaneous injections of 0.05

[5] We thank Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases for determinations of CF and HI tests on these mice.

ml. of undiluted material, 15 developed salivary-gland tumors, with a latent period of 1.5 to 3.5 months.

Cultures.—The newborn mice were killed by ether anesthesia and vigorously scrubbed with 70 percent ethanol. Under a dissecting microscope, the salivary glands were excised *en bloc*, with the use of sterile instruments. In the first three culture series, an attempt was made to obtain parotid-gland tissue along with the submaxillary and sublingual glands by including the soft tissues adjacent to the posterior wall of the external ear canal. In the fourth series, however, only submaxillary and sublingual glands were taken.

Each litter of donor mice was divided equally for use in test and control cultures. Four sets of cultures were set up, the first and third using 1 litter of 6 mice each, the second using 2 litters of 6 mice each, and the fourth using 1 litter of 7 mice. The salivary-gland tissue for the test cultures was exposed to the agent by immersing the excised glands immediately in the agent preparation in a sterile petri dish. Tissue for the control cultures in the first 3 sets was similarly immersed in the tissue-culture medium containing no agent. In the fourth set, tissue for the control cultures was immersed in the filtrate of uninfected P388 D_1 cells described. All tissues were cut into explants of approximately 1.0 mm. in diameter and were allowed to stand in their respective preparations at 20° C. for 2 hours before the cultures were prepared.

Gelatin sponge-matrix cultures were used throughout, after the technique of Leighton (*8*). The modification used here has been described in detail previously (*7*). Six to 12 explants were placed in each Leighton tube, clotted to the sponge with chicken-plasma clot. Since the explants were not washed before they were placed in culture, and small amounts of the agent preparation were necessarily introduced into the cultures of the test group, actual exposure to the agent presumably continued for a time during incubation of the cultures.

The 4 series of cultures included a total of 26 test cultures and 24 controls. Feeding intervals were determined on the basis of *p*H change.

On the 5th, 12th, 19th, 26th, 33d, and 42d days, one culture each from the test and the control groups of the first series was fixed in Zenker-formalin, serially sectioned, and stained with hematoxylin-eosin. The second and third series were allowed to continue to 48 days, at which time 6 test and 6 control cultures were fixed, serially sectioned, and stained. The remaining 8 test and 7 control cultures of these series were transplanted under the kidney capsules or subcutaneously in C3Hf/Bi mice, the strain of origin of the salivary-gland tissues. These mice had received 300 r of total-body X radiation 2 days prior to receiving the cultures.

The fourth culture series consisted of 6 test and 5 control cultures (1 control culture was lost to a contaminant). Three test and 2 control cultures were fixed and serially sectioned at 29 days and the remaining 3 test and 3 control cultures were handled similarly at 32 days.

RESULTS

Living Cultures

Two differences between the test and control groups of each culture series were noted. The first was a relatively lower rate of *p*H change in the test cultures as compared with controls. This difference became evident in each series by the end of the 1st week and continued throughout the duration of the culture period.

The second difference was probably related to the first and consisted in markedly smaller populations of macrophages in the PTA-exposed cultures. The difference in this respect, too, was apparent after the 1st week of culture, and, among those cultures carried for a month or more, only those in the control groups showed large numbers of macrophages adherent to the glass. Macrophages were virtually absent on the glass in the cultures exposed to PTA, though fibroblasts were fairly numerous in a few. This finding is consistent with our previous observation that cultures of normal macrophages infected with PTA decline in population more rapidly than do similar cultures not exposed to this agent (*6*).

Fixed Cultures

At the 5th and 12th days, no significant morphologic differences could be detected between the exposed and unexposed salivary-gland tissues. In fact, at both these times, as illustrated in figures 1 to 4, there was somewhat better survival of salivary-gland tissue in the control than in the test cultures. This is presumed to be only a chance occurrence, because no qualitative difference between the test and controls could be seen. As was to be expected in this type of culture, central necrosis of the explants occurred in the early periods after isolation. Also, as shown in figures 3 and 4, cultures at the 12-day interval showed loss of the differentiated features of salivary-gland acini, with varying degrees of conversion into tubular structures. The fields shown in figures 3 and 4 presumably represent the type of surviving cellular elements from which the structures seen at later intervals were derived.

At the 19th day and at all succeeding time periods it was readily possible to distinguish the exposed cultures from unexposed ones on the basis of the microscopic characteristics. Figures 5 and 6 illustrate control and test cultures at 19 days, and figures 7, 11, 13, and 16 illustrate control cultures at later intervals. Explants in the controls showed only small nests of viable tissue made up of atrophic tubular structures apparently derived from acini. Explants in the exposed cultures (figs. 6, 8, 10, and 12) contained generally larger and more vigorous-appearing cell groups that no longer conformed to the pattern of atrophic or metaplastic salivary-gland tissue. Instead, the cells within the tubular structures displayed a tendency to pile up in clusters or to form solid, branching cords. In some instances, giant lumens were formed (fig. 14). The geometric patterns were irregular and more frequently branched than in

the residual duct-acini systems of the controls. A comparison between small tumors *in vivo* and the changes in the exposed tumors *in vitro* can be made from figures 9 and 10, and figures 21 and 22.

Cytologically, cells of the exposed salivary glands showed changes of similar nature at all the time intervals observed, and these changes are therefore described together. Cells of the salivary-gland epithelium in the cultures exposed to the virus were generally larger than those in control cultures, and anisocytosis was much more pronounced (figs. 17 and 20). Whereas in the controls the cells were uniform in both nuclear and cytoplasmic dimensions, in the test cultures some nuclei were 3 or 4 times larger than those of adjacent, apparently unaltered cells. Nuclei in the exposed cultures, but not in the controls, rather frequently showed an area of decreased density of the chromatin, and occasional examples showed the presence of one, or rarely, two sharply circumscribed clear spaces, resembling intranuclear vacuoles within the nucleus. These intranuclear clear spaces were unstained by either hematoxylin or eosin, and sometimes occupied all but a densely hematoxylinophilic shell of the nucleus (figs. 17, 18, and 21). Rarely, (figs. 15, 17, and 20) a nucleus in the test cultures contained a large nucleolus-like body surrounded by a nonstaining zone between it and the marginated nuclear chromatin. Such nucleolus-like bodies stained with an amphophilic tint. The nucleoli themselves were moderately enlarged in many cells, which made it difficult to exclude the possibility that the bodies just described may be nucleolar derivatives.

At all time intervals after 12 days, mitotic figures were much more numerous in the exposed cultures than in controls. At 48 days, the mitotic index for exposed cultures was 9.8 as compared with 1.1 in the controls (5,000 nuclei counted in each group). For the most part, mitotic figures appeared normal in test as well as in control cultures and more precise study would be required to establish a significant qualitative difference.

Evidence of cell necrosis was usually present in the same cell groups in which mitoses were found in the test cultures (figs. 10 and 14). Nuclear debris was commonly present in the lumens of gland and ductlike structures, and necrotic cells often could be distinguished within viable cell groups. The proximity of nuclear debris to groups of cells with vacuolated nuclei led to the impression that cells exhibiting the intranuclear vacuoles were in the process of dying. In nuclei containing the largest of these vacuoles, the chromatin had generally lost its reticular character and was condensed into a dark rim. Nuclear rupture was a sequel of this stage, with fragmentation of the condensed chromatin.

Although the intranuclear change described is less commonly seen in salivary-gland tumors induced by this agent *in vivo* than in the salivary-gland explants *in vitro*, it does occur. This intranuclear lesion may be found not only in salivary-gland tumors and tumors of the submucosal glands of the upper respiratory tract, but also in the early thyroid tumors (fig. 22) and in the epithelium of the characteristic renal tubular lesion

associated with the parotid-tumor virus. In fact, in our experience, intranuclear clear spaces are more prominent in the latter two locations than at any other location. These nuclear lesions have been previously referred to by Stewart *et al.* (*9*) as "ballooning" of nuclei, and they appear to be similar to those studied cytochemically by Love and Rabson (*10*) in P388 D_1 cultures infected with PTA.

The morphologic transformations described were observed in 15 of the 16 test cultures older than 12 days, and in none of the 17 control cultures. In a single culture among the test group, no surviving salivary-gland tissue could be found. In addition to the changes already mentioned, 2 cultures showed proliferations that appeared to be within salivary-gland ducts, rather than in acini. One of these is illustrated in figure 19, in which it may be seen that the cells in more central portions of the duct-like structures resemble squamous epithelium. No intercellular bridges could be demonstrated, however. Changes similar to those shown in figure 19 were not observed in control cultures, but neither have we seen intraductal origin of tumors in the salivary glands *in vivo*. The significance of this type of change therefore remains uncertain.

No definite cytologic differences have been found between surviving nonepithelial cells, such as fibroblasts or endothelium, in the test and in the control cultures. However, it was rather consistently noted that stromal-cell elements were decreased or absent in the vicinity of the altered epithelium of the exposed cultures, by comparison with the controls (figs. 7, 8, 11, 12, 13, 14, 16, and 17).

Results of Transfer of Cultures to Mice

A preliminary report of the results of transfer of cultures to animals is included here because of their apparent significance to basic questions concerning host-agent relationships in this system. More time is necessary before the data on this and additional experiments will be completed.

Thirty-nine C3H/BiLw mice received material from test and control cultures of series 2 and 3. All were animals between 1 and 2 months of age, and all had received 300 r of total-body X radiation 2 days before receiving the cultures.

Eight of these animals received the gelatin sponges and their contained explants from cultures exposed to PTA. Seven received comparable material from the controls. Each sponge was cut lengthwise into 2 approximately equal strips, and one strip was placed under the left renal capsule and the other subcutaneously in the right axilla of the same animal. Pentobarbital anesthesia was used for the renal transfers.

In addition, the cells adherent to the glass of 11 test cultures and 13 control cultures were detached with a rubber policeman and injected subcutaneously into one animal per culture.

None of the 24 mice receiving cells removed from the glass of either the test or control cultures have shown visible or palpable evidence of tumors during a 4-month observation period. Three of these mice died

within 2 weeks of the exposure to X radiation, and showed multiple hemorrhages in the brain.

None of the 7 mice that received sponges from control cultures have shown any visible or palpable tumors during a 4-month observation period.

Two of the 8 mice receiving sponges from PTA-exposed cultures died, one immediately postoperatively and the other from radiation effects 9 days postoperatively. Of the remaining 6, 5 have developed bilateral salivary-gland tumors in 2.5 to 4 months after receiving the cultures. These mice were autopsied and found to have tumors of the thymus gland, hair follicles, bones, thyroid gland, lacrimal glands, and salivary glands. All these tumors were histologically similar to those previously reported in the organs of mice that had received parotid-tumor virus at birth (*6*).

In none of the autopisied tumor-bearing mice was there gross or microscopic evidence of tumor of any kind under the renal capsule or subcutaneously at the site of culture transfer.

DISCUSSION

The microscopic changes were consistently limited to the cultures exposed to agent and were faithfully reproduced in each of the four separate culture series. Regardless of how one may wish to classify the salivary-gland lesions *in vivo*, it has been demonstrated that morphologically similar lesions can be induced under the influence of PTA *in vitro*.

It would be difficult to appraise accurately certain minor differences between the lesions observed *in vivo* and *in vitro*. For example, it appears that in tissue culture the cytolytic effect of the agent is relatively more prominent than *in vivo*. However, both *in vitro* and *in vivo*, the proliferative response dominates.

This observation may have serious implications concerning the applicability of plaque-forming techniques to the isolation of clonal strains of PTA by the methods used by Stewart *et al.* (*11*) and by Sachs *et al.* (*12*). These workers used mouse-embryo cultures, composed of many different cell types mingled together in monolayers. If certain virus-infected cells of such cultures were capable of proliferation, as suggested by the observations in our system, then it would be difficult to be certain that virus fished from a particular plaque was not mixed with virus from adjacent viable, but infected, cells. The importance of using a pure cell strain reacting uniformly to the virus is thus emphasized for plaque-forming techniques with this agent.

It should be recognized here that a direct, morphologic response to an agent-containing preparation has been observed in at least *one* site at which tumors have been observed in animals receiving PTA. It is entirely possible that at some sites other than salivary gland the action is indirect. There is also the possibility that influences such as hormonal ones may be superimposed on the initiating effects of the agent, either before or after it has had access to the cell. This point is of interest

particularly with regard to the mammary tumors, inasmuch as these previously occurred in approximately a 3-to-1 ratio in females over males (*6*).

It must also be recognized that, although this experiment excludes an intermediate function of other *organs* in the morphologic transformation of salivary-gland epithelium, it does not exclude possible intermediation by other *cells*, such as macrophages or fibroblasts, for example. Many cell types in addition to salivary-gland epithelium are obviously present in the explants of salivary-gland tissue placed into cultures. These include, besides macrophages and fibroblasts, vascular endothelium, adipose cells, ganglion cells, neural sheath cells, and probably all types of blood cells. Conceivably, infection of one of these cell types might be prerequisite to induction of the changes in the epithelium.

The appearance of multiple primary tumors in X-irradiated hosts receiving the PTA-exposed sponge cultures was not entirely anticipated and appears to be of considerable import. Interpretation is difficult at this time. It seems most likely that irradiation modified the immune response of these mice to the extent that agent carried in the cultures was able to infect and induce tumors in the same organs usually affected by inoculation only during the newborn period. If this proves to be true, the concept that "age" *per se* is responsible for the difference in susceptibility between newborn and older mice may require drastic revision.

The fact that tumors have not developed at the sites of transplant of PTA-exposed cultures is difficult to explain. It suggests that the morphologic changes observed *in vitro* may not be completely equivalent biologically to the similar changes observed *in vivo*, or that a longer period in tissue culture may be required for evolution of the expected biologic characteristics. Evidence obtained earlier has indicated that some of the tumors associated with this agent do not behave on transplantation as do most "spontaneous" neoplasms or neoplasms induced by nonviral oncogenic agents. Thus, only 5 of 7 previous attempts to transplant parotid tumors were successful, with the use of large inoculums. We have not succeeded in transplanting any of 14 thymic tumors, associated with this agent, to nonirradiated hosts of the proper genotype (*6*).

Through modifications of the technique used here, it may prove possible to develop systems comparable to those used by Rubin and colleagues (*13*) in studies on the mechanism of action of Rous virus *in vitro*. Further, the results in this experiment suggest that it may serve as a model on which to pattern investigations on the activity of PTA or other agents on human tissues *in vitro*. The availability of viable human tissues from stillborn infants and spontaneous abortions makes this approach practicable, as has been demonstrated in the investigations of Bang (*14*) and others.

ADDENDUM

Since submitting this manuscript for publication, the authors have observed the appearance of the characteristic morphologic changes in

salivary-gland tissue in culture within 14 days after explantation and infection. This reduced time interval for the appearance of changes was observed in a gelatin sponge culture set up in a tube of P388 D_1 "carrier" cells chronically infected with and producing PTA, as previously described (*6*). Samples of the culture were removed and examined by serial sections at progressive intervals. Changes comparable to those described in this work were first noted at 14 days, and included an increase of mitotic rate.

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PLATES

PLATE 111

FIGURE 1.—Section of 5-day culture of salivary gland, not exposed to parotid-tumor agent (PTA). Central portion of explant is necrotic, while glands on periphery remain viable. Gelatin sponge matrix is at *lower left.* × 150

FIGURE 2.—Section of 5-day culture of salivary gland exposed to PTA. Necrosis may be more extensive here than in control in figure 1, but differences are not significant. × 150

FIGURE 3.—Section of 12-day control culture of salivary gland. Necrosis has become more extensive and cells of the residual acini are atrophic. × 150

FIGURE 4.—Section of 12-day culture of salivary gland exposed to PTA. Necrosis is even more extensive than in control, but there are no specific changes. × 150

FIGURE 5.—Section of 19-day control culture of salivary gland. Small groups of acini survive, but cells are atrophic. × 150

FIGURE 6.—Section of 19-day culture of salivary gland exposed to PTA. Cells are beginning to fill the lumens of acini and ductules, which are producing "buds" that extend into the gelatin sponge. Individual cells are larger and more basophilic than those of controls, and nuclei are larger and sometimes contain large, clear areas. *Note* large nucleus at *upper right,* with marginated chromatin and central nucleolus-like body. × 150

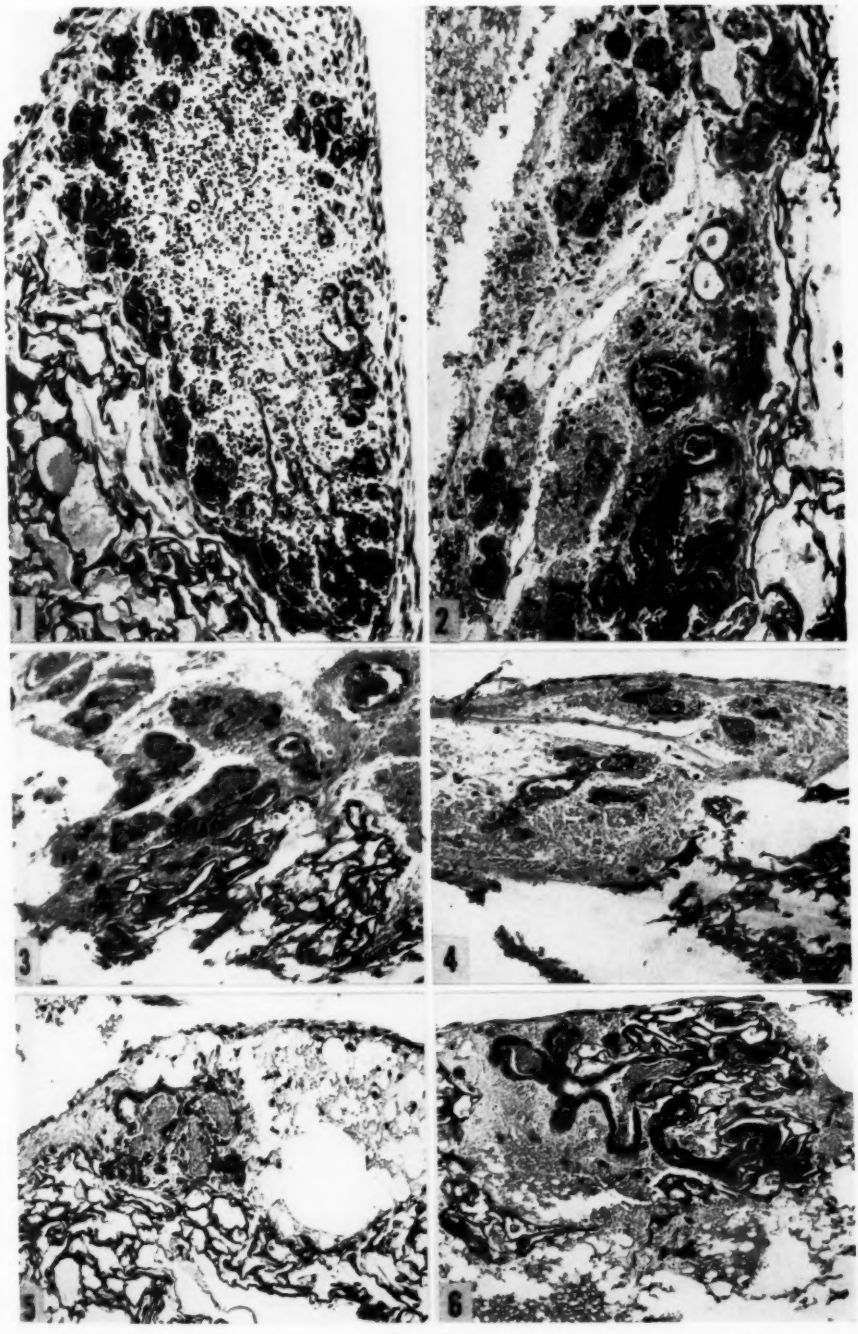
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PLATE 112

FIGURE 7.—Control culture of salivary gland at 26 days. Cells of surviving ductlike structures appear to be in good condition, but are not proliferating. × 150

FIGURE 8.—Twenty-six-day culture of salivary gland exposed to parotid-tumor agent (PTA). Cells appear vigorous, are filling the glandular lumens, and are proliferating to form small, lateral extensions. × 150

FIGURE 9.—Section of small, developing tumor in lobule of submaxillary gland of (C3Hf × AKR)F_1 mouse that had received PTA at birth. *Compare* with changes *in vitro* illustrated in figure 10. × 130

FIGURE 10.—Twenty-nine-day culture of salivary gland exposed to PTA. *Note* high degree of cellularity and filling of glandular lumens by proliferating cells. Clear spaces within nuclei are visible in areas at *upper right*. Necrosis is present at *mid-left*. There are small, solid nests of proliferating cells in *lower center*. × 130

FIGURE 11.—Control culture of salivary gland at 33 days. Ductlike structures persist, but show no proliferative activity or cellular alterations comparable to those in test cultures. × 150

FIGURE 12.—Thirty-three-day culture of salivary gland exposed to PTA. Distortion of glandular pattern and vacuolation of nuclei (*lower right*) are present, though proliferation is not marked. *Note* disappearance of stromal cells as compared with figure 11. × 150

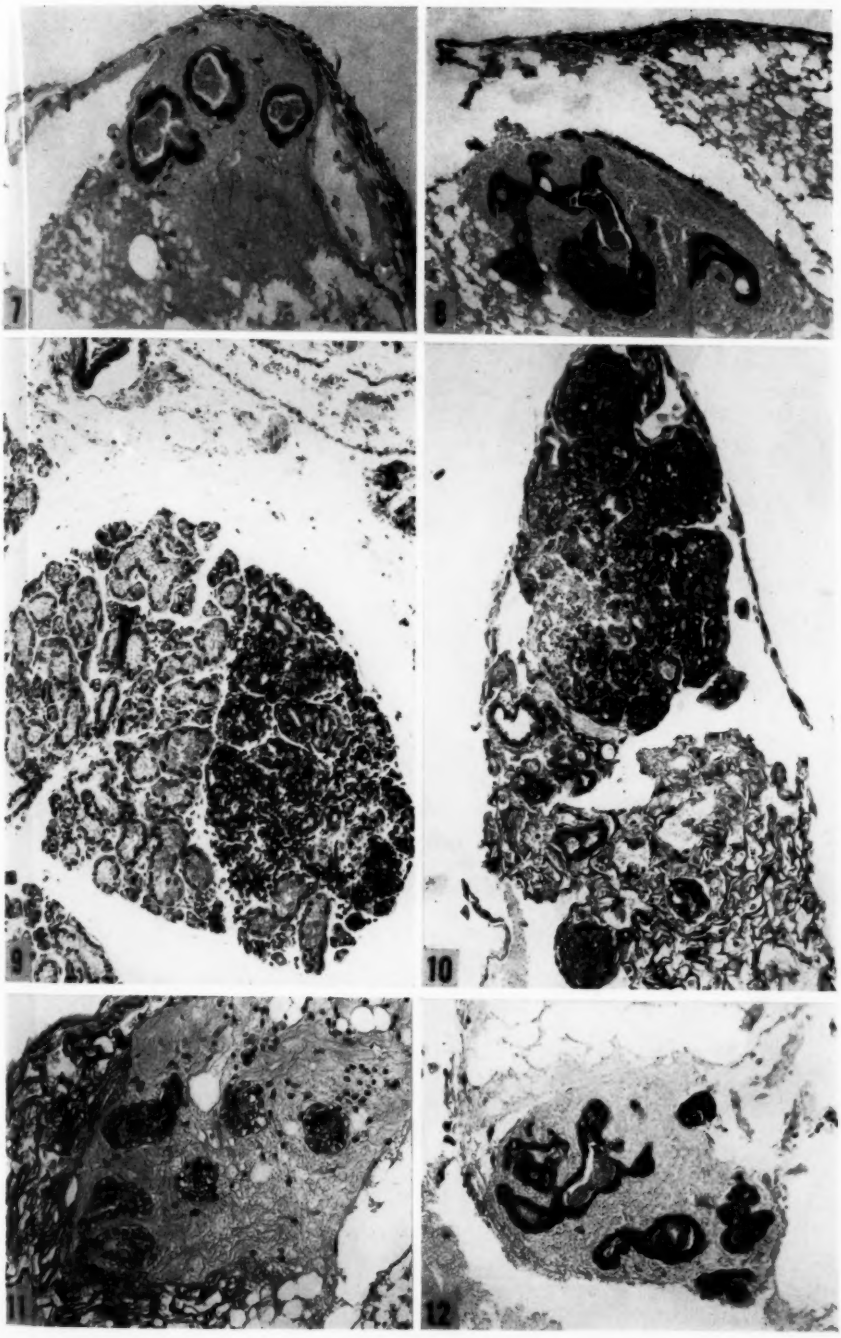
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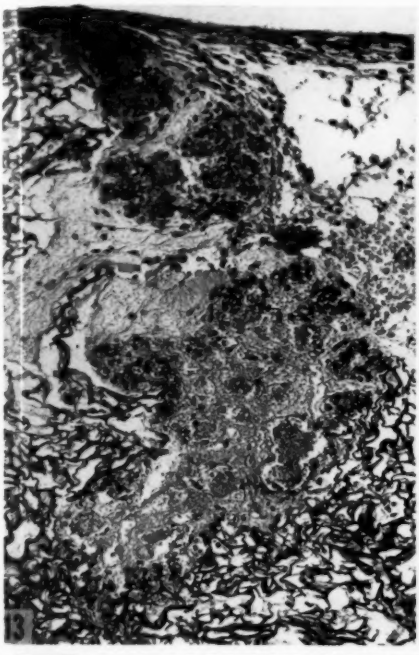
PLATE 113

FIGURE 13.—Control culture at 29 days. Cells of surviving ducts and acini appear atrophic. × 165

FIGURE 14.—Twenty-nine-day culture infected with parotid-tumor agent (PTA). Centers of proliferating cell masses have become necrotic, leaving cystlike structures in which proliferation continues on periphery. Mitotic figures at *upper right* (*arrows*). × 165

FIGURE 15.—Section adjacent to that shown in figure 14. *Note* variation in size of nuclei, and two adjacent cells in mitosis at *lower right* (*arrow*). Near *center* of field is a nucleus with a large, amphophilic body surrounded by a clear zone (*arrow*). × 420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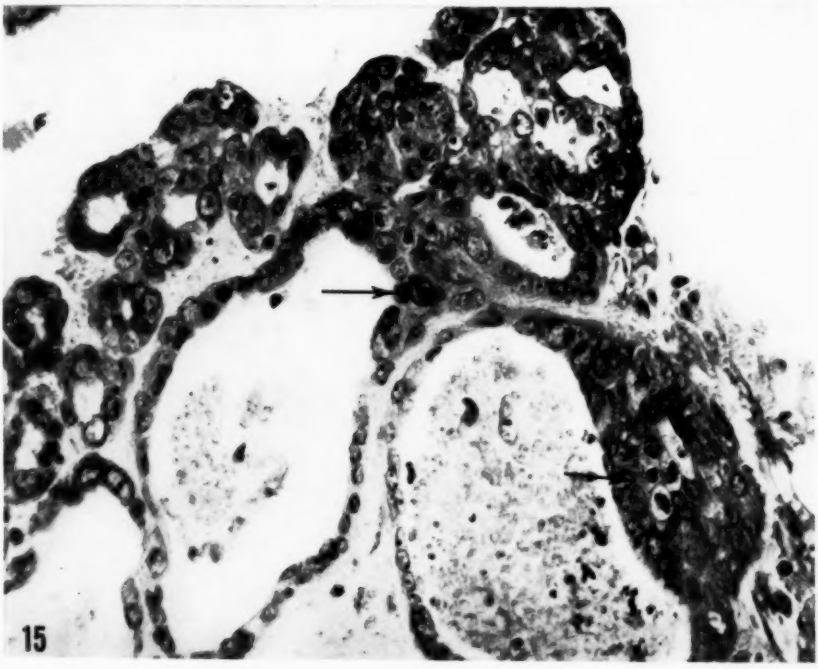
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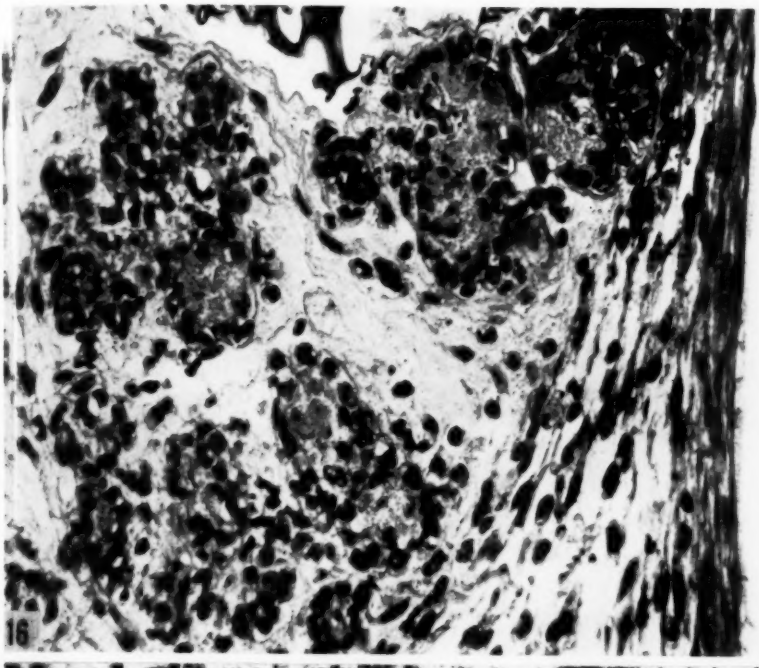
PLATE 114

FIGURE 16.—Higher magnification of 29-day control culture shown in figure 13. *Note* maintenance of normal glandular pattern and uniformity of cells. × 520

FIGURE 17.—Higher magnification of 29-day infected culture shown in figure 10. *Note* necrosis in *upper* portion of field, mixture of small and large cells at *lower left*, and solid masses of large cells at *lower right*. Cells with vacuolated nuclei are prominent at *left center* and *upper right* (*arrows*). Cell containing large, intranuclear, amphophilic body is indicated by *arrow* at *lower left*. Stromal cells are absent here, as compared with a relatively vigorous stroma in control (fig. 16). × 520



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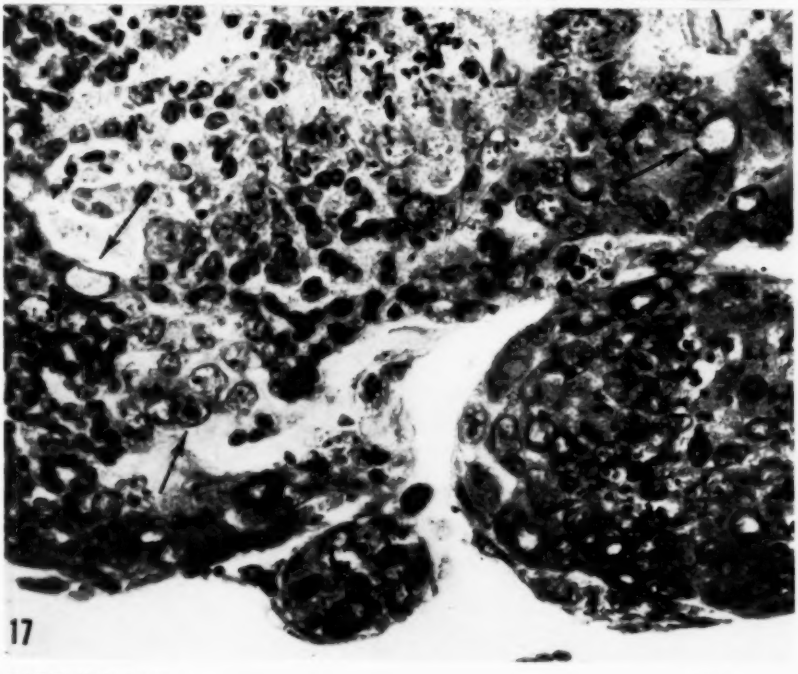

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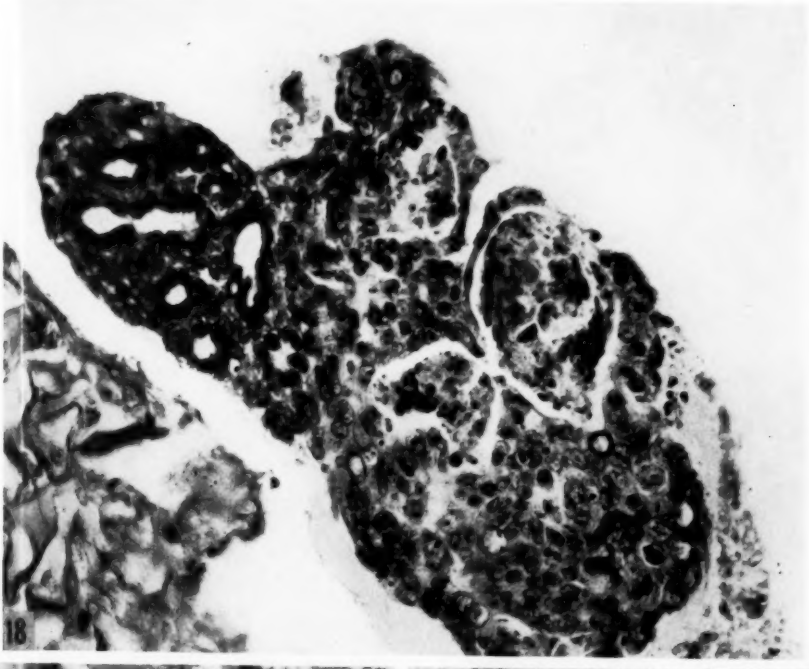
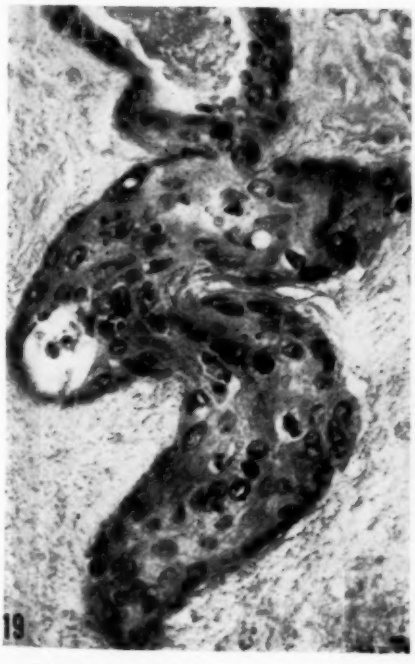
PLATE 115

FIGURE 18.—Twenty-nine-day culture of salivary gland exposed to parotid-tumor agent (PTA). Nearly all cells in this field appear to have undergone alteration. Small, dark cells forming tubular structures at *upper left* show less extensive change than those forming solid cell masses. *Note* loss of stromal elements. × 420

FIGURE 19.—Forty-eight-day culture of salivary gland exposed to PTA. Altered cells here appear to be forming a ductlike structure and resemble squamous cells, though no intercellular bridges are demonstrable. × 340

FIGURE 20.—Two acinar structures in a 48-day culture of salivary gland exposed to PTA. Only a portion of cells show enlarged nuclei and intranuclear changes. At *middle right* (*arrow*) a nucleus contains a large amphophilic body surrounded by clear zone. × 780





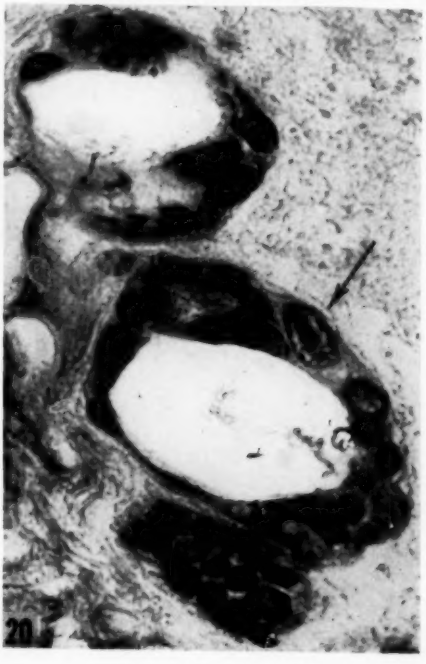
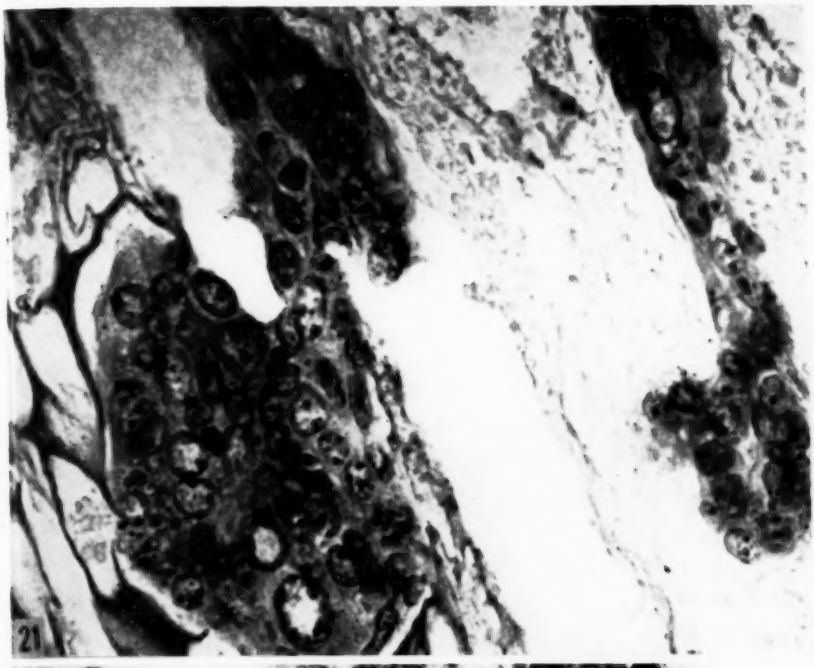


PLATE 116

FIGURE 21.—Forty-eight-day culture of salivary gland exposed to parotid-tumor agent (PTA). All cells in this field show alteration. *Note* solid cords and masses of cells with relatively large nuclei and scant, poorly demarcated cytoplasm. Several nuclei show vacuolation. × 780

FIGURE 22.—Small, early tumor nodule in thyroid gland of RF mouse inoculated with PTA shortly after birth. *Note* striking similarity of cells in this lesion to those of culture in figure 21, despite the fact that they originated from different organs. × 780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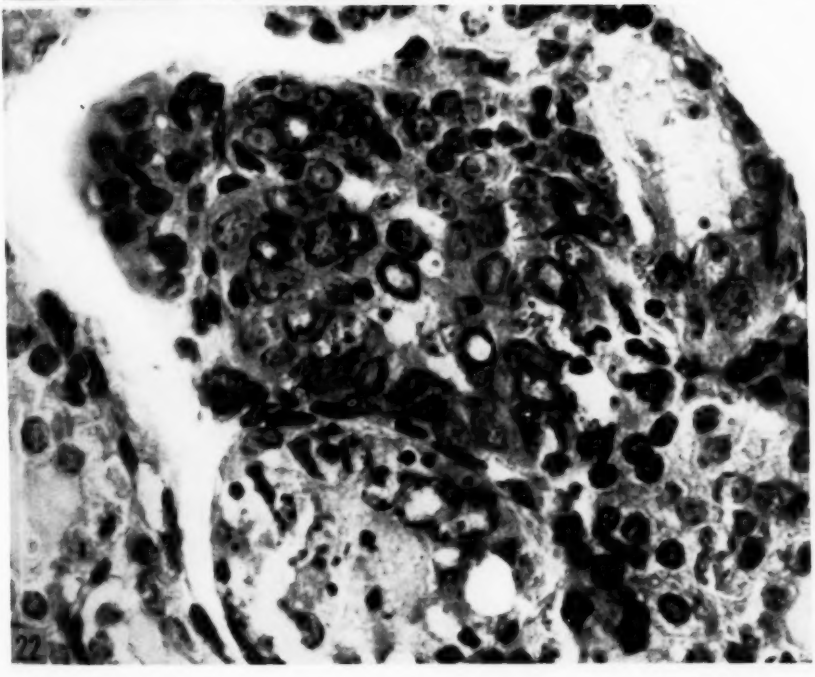
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Relative Status of Parity and Genetic Background in Producing Human Breast Cancer [1, 2]

MADGE T. MACKLIN, *M.D.*,[3] *Department of Medicine, Ohio State Medical School, Columbus, Ohio*

SUMMARY

There is no significant difference in the frequency of breast cancer in childless women who are either single or married. Nulliparous ever-married women have no less breast cancer than nulliparous single women. The highest frequency of breast cancer was found in nulliparous women, with decreasing frequency of the disease as the number of pregnancies increased. The genetic or familial factors presumably present in some of the close relatives of women with breast cancer appear to be as potent as the parity status in determining the presence of breast cancer. The effects of the two sets of factors appear to be additive, so that nulliparous women closely related to a woman with breast cancer have more breast cancer than nulliparous women found in the general population. The underlying factors connected with parity may be the primary exogenous factors, but being inevitably associated with physiological changes found in parous women they have not been identified. Socioeconomic factors are so intimately correlated with size of family, and whether a woman is single or married, that the association between socioeconomic rating and breast cancer may merely reflect the association between parity status and breast cancer.—J. Nat. Cancer Inst. 23: 1179–1189, 1959.

MOST STUDIES dealing with causes of human breast cancer have been concerned with either the genetic or the nongenetic factors in its development, but not with the effects of both factors simultaneously. The only nongenetic factors which, so far, have been definitely correlated with human breast cancer by almost all workers are (1) socioeconomic status and (2) marital status. Single women and those of higher socioeconomic rank have a significantly higher mortality rate from breast cancer than married women and those of lower socioeconomic range. Dorn (*1*) stated that the higher rates for single women held for all ages and that the excess in mortality rate increases with increasing age. Also, United States census data show that widowed and divorced women have a higher mortality rate from breast cancer than married women, though

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[3] *Present address:* 37 Gerrard Street, London, Ontario, Canada.

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a lower rate than single women. Most students of the genetic aspect of human breast cancer have concluded that genetic factors contribute [Jacobsen (*2*), Penrose *et al.* (*3*), and Woolf (*4*)]. The purpose of the present investigation is to compare the relative effects of the genetic or familial factors and the known nongenetic factor of parity in the same population. If both are important, and if the factors have an additive effect, the highest percentage of breast cancer should occur when both determinants are acting simultaneously. As one or the other factor decreases, the amount of breast cancer should be decreased. Socioeconomic factors will not be discussed for two reasons: (1) That practically all the probands with breast cancer were clinical patients and would certainly not be classed in the highest socioeconomic range. Neither were they in the lowest range socially or economically. (2) Socioeconomic status and parity are so closely bound that so far it has not been possible to separate the two. The higher the social and economic rating, the fewer the children, and *vice versa*. To my knowledge no study has been made that separates the 2 factors. Such a study would be worth while, if it included women, with the same number of children, separated into their various socioeconomic classes (as far as this has any real meaning) and compared the frequency of breast cancer for the various groups.

The evidence will be presented in three steps: First, whether it is the single state *per se* or the childless state, which accompanies the unmarried condition in most instances, that predisposes to breast cancer. Second, whether with an increasing number of children there are decreasing percentages of breast cancer, or whether the maximum protective effect is achieved with the first child. Third, an assessment will be made of the relative status of the genetic or familial factors and the nongenetic factor of parity. It is not to be assumed that this is the only nongenetic factor operating. It is, however, associated with marital status, the one other exogenous factor most frequently discussed.

MATERIAL

This study is based on material collected as part of a larger project to ascertain whether there was any evidence of a genetic basis for human breast cancer, and of a milk agent in man. The methods used in collecting the probands with breast cancer and the controls have been discussed fully by Macklin (*5*), together with the possible sources of bias and the extent to which they were avoided, and will not be repeated here. There were 295 white women with pathologically diagnosed breast cancer, whose families were given the designation P1, 300 white patients with similarly diagnosed cancers of organs other than the breast, and 246 white women with no cancer at all at the time of interview. The last two groups exhibited a similar amount of breast cancer and were grouped together. These will be referred to here as the controls. In the earlier paper (*5*) by using mortality rates, it was shown that relatives of P1 pro-

bands had significantly more breast cancer than the population of Ohio with the same age range and period of death distribution, but that the relatives of the control series showed no significant difference in breast cancer from the population of Ohio. For both breast-cancer probands and controls, the relatives studied included the grandparents, parents, uncles, aunts, sibs, and first cousins. All death certificates were checked whenever possible, and no instance was recorded as breast cancer unless it was stated on the death certificate.

The marital status of each female was recorded as either single or married, meaning in this instance, ever-married. This latter group was composed of the women who were widows, or divorced, or still married at the time of their death. In addition, the number of living children that each woman had was recorded, and, if possible, the number of miscarriages. The latter were not always known, especially for women who could not be interviewed or written to personally, so that the live births constituted the basis for deciding the parity status of the women.

When the data on these families were complete, a new project for the study of gastric and large-bowel carcinoma was started. An additional 200 families with these cancers were interviewed and the data collected up to the time of analysis. Among the relatives of these patients were women who had died with breast cancer. To increase the size of the sample, the nulliparous married and single women were added to the control group for the first table. They were a homogeneous sample with the rest of the relatives of cancer probands, both in age distribution and frequency of breast cancer. Tests of difference between these two samples gave P values in each instance between 0.90 and 0.80, respectively.

Frequency of Breast Cancer in Single and Married Nulliparae

Most of the studies on the frequency of breast cancer among single and married women are based on death records which do not state how many children, if any, the married women had. Such information can be gleaned only by a study of individuals. The first point to be established is whether the marital state, *per se*, without accompanying children, confers some degree of immunity to breast cancer. If ever-married nulliparae do not differ from single women in the frequency of breast cancer, these two classes of women can be grouped in one category.

Widowed and divorced women are said to have a higher mortality rate from breast cancer than married women, but a lower rate than single women. No cognizance will be taken of these two classes, in the discussion, for the following reason. All such women have at one time been married and can be assigned, therefore, to the group of women who have had marital experience. Those who were childless can be designated as nulliparous women who had at some time been married, though they may have been widowed or divorced at the time of their death from breast cancer. To show how meaningless such a division into widowed or divorced would be, unless some factors were involved other than the

altered marital status, one need consider only a few possibilities. A married woman may have developed her breast cancer while still married, but may have died from it several years after she was widowed or divorced. It is obvious that the marital status listed on her death certificate is not the marital status she had at the time she developed breast cancer, and any listing other than married, in connection with breast cancer, is misleading. She may have been divorced, then remarried, and so be listed as married, or she may have remarried and been widowed before she died and be listed as widowed. The one circumstance that in some way might be associated with this higher mortality rate from breast cancer is the number of children she had. Therefore, all women who had been married at some time are classed in that group, and further separated into groups by the number of children they had.

Age Distribution of Single Women and Ever-Married Nulliparae in P1 and Controls

Before estimating the relative frequencies of breast cancer in single and nulliparous ever-married women, the age distribution in the two groups must be considered. It has been pointed out that the age distribution of single and married *living* women is quite different. This is understandable, because comparisons begin in the United States census at 15 years of age, at which time most of the women are single. The women considered in this paper are *deceased*, and the youngest age is 30 years. Actually, the age distribution of single and ever-married women, living or dead, in the general population need not be considered here; it is the age distribution of the sample under discussion that is important. The single and ever-married nulliparae of P1 and the controls were separated into their respective categories and were further subdivided into quinquennial age groups beginning with age 30 to 34 years and ending with 80+. The age distribution of single and ever-marrried women of P1 was so similar that the probability of obtaining even greater disparity in a random sample was > 0.80 ($X^2 = 5.87$, d.f. $= 10$). Similar results were obtained for the childless relatives of the controls, who were either single or married. The value of P for the controls was > 0.50 ($X^2 = 8.30$, d.f. $= 10$).

The two groups of relatives were then inspected for the number of breast-cancer cases in the single and ever-married nulliparae. There was no significant difference in the amount of breast cancer in the single and married women of either group (table 1). For P1, $X^2 = 2.39$, d.f. $= 1$, $P > 0.10$. For the controls, $X^2 = 0.34$, d.f. $= 1$, $P > 0.50$.

There is always the criticism that the sample used is biased in some way. There were available 1,474 death certificates of white women, who died in 1957, in Franklin County, Ohio, for whom the number of children, the marital status, and the cause of and the age at death were known. The number of children had been obtained by writing to the informants on the death certificates. The data were separated into

TABLE 1.—Number of women and number of breast cancers in deceased single and nulliparous married women in two sample populations*

Population	Single		Ever-married	
	Number of women	Number of breast cancers	Number of women	Number of breast cancers
P1	93	18 (2)†	121	14
Controls	277	19	317	18

*The total controls in table 1 is greater than nulliparous controls in table 4. For explanation *see* text.

†Reported as breast cancer but not verified by official record.

quinquennial age groups from 30 years through 90+. The records of women who had remained childless were removed, and this group was again divided into single women and those who had been married. The number of breast cancers was noted in each group. There were 237 ever-married childless women with 22 breast cancers, and 180 single women with 12 breast cancers. The percentages of the ever-married women in each age group were determined, and the proportionate number of single women, who should have been in the same age group, was calculated. The proportionate number of breast cancers which should have occurred in the single women of that age group was then found. Thus 20 single women, of whom 1 had breast cancer, were in the group between 70 and 74 years of age. Proportionately there should have been 33.4 women in that group with 1.67 breast cancers to be comparable to the married group. When the total number of expected breast cancers was derived in this manner, there should have been 12.6 breast cancers in place of the 12 found. From this group of women taken routinely from the death records of Ohio, and followed for the number of their children, confirmation of the findings is shown in table 1, based on the samples selected for the breast-cancer study. It appears reasonable to conclude that childless women, whether married or single, do not differ significantly in the degree to which they succumb to breast cancer. They can, therefore, be treated as a class of nulliparous women, regardless of their marital status.

Decreasing Risk With Increasing Number of Pregnancies

Denoix and Moine (*6*) showed that the mortality rates from breast cancer decreased as the number of pregnancies increased. The author's data were examined to see whether the percentage of deceased women with breast cancer declined as the number of pregnancies increased.

All deceased relatives of P1 and controls, minus the relatives of the 200 probands in table 1 for whom the parity status was known, were grouped into 6 parity ratings. These are shown in table 2, together with the number of women and the number and percentage of breast cancers in each group. The percentage of breast cancers decreased as the number of pregnancies increased, though the largest difference occurred between

the women with no children and those with 1 and 2. The difference between each parity group and the following one was not significant, though the difference between the women with no children and those with children was highly significant ($X^2 = 40.96$, $P<0.001$).

The number of women with no children in table 2 does not agree with that in table 1 for the reasons given. The childless women in table 2 were compared with women having 1 and 2, 3 and 4, etc., for similarity of age distribution when placed in quinquennial age groups from 30 years on. The groups were all similar in age distribution so that the percentages of those with cancer could be calculated as if they were a homogeneous group. It will be noted that the percentage of breast cancer decreases with increasing number of children, and it appears that the greater the number of children, the higher the degree of immunity from breast cancer.

Again, a test of this conclusion was made with the data on the 1,474 women who died in Franklin County in 1957. The women were divided into quinquennial age groups and according to number of children. The number of women in each group in the table was adjusted to comprise the same percentage for that group as existed in the nulliparous married women already referred to. The proportionate number of cancers that should have existed was then calculated as for the single women. Table 3 shows the number of women in each parity rating, the number of cancers actually found, and the number there should have been if the age distribution was identical with that of the nulliparous married women. The age distribution was practically identical in each parity rating with that of the nulliparous married women, as evidenced by the very slight change in the number of breast cancers found and expected. Since the close similarity in age distribution had been determined, the percentages of breast cancer were determined for each parity group. To make the table comparable with table 2, the women with 1 and 2, 3 and 4 children, etc., were grouped and the percentages computed. As in table 2 there is a decreasing percentage of breast cancer as parity increases, except for minor variations. This group taken routinely from the Ohio death certificates is in agreement with the sample chosen for the breast-cancer study.

TABLE 2.—Frequency of breast cancer in deceased women of differing parity

Number of children	Number of women	Number of breast cancers	Percent with breast cancers
0	643	60	9.5
1, 2	998	45	4.5
3, 4	1048	46	4.4
5, 6	779	23	3.0
7, 8	495	14	2.8
9+	491	12	2.4
Total*	4454	200	4.5

*The totals here do not correspond to those in table 4, since these include cousins, who are excluded from table 4.

TABLE 3.—Frequency of breast cancer in deceased women of differing parity who died in Franklin County in 1957

Number of children	Number of women	Number of breast cancers	Number of breast cancers expected*	Percent with breast cancers	
0	417	34	34.0	8.2	
1	219	19	21.1	8.7	7.2
2	256	15	15.3	5.9	
3	162	9	8.5	5.6	5.4
4	116	6	5.2	5.2	
5	88	3	2.9	3.4	2.6
6	63	1	0.7	1.6	
7	45	1	1.2	2.2	3.4
8	41	2	1.8	4.9	
9+	67	1	0.8	1.5	1.5
Total	1,474	91	91.5		

*For method of calculating the expected number *see* text.

Comparison of Effects of Genetic and Parity Status in P1 and Control Relatives

Although the fact that a woman does not have children may sometimes be dependent upon genetic factors, it is true that frequently her parity status is associated with environmental conditions, such as economic and social status, her opportunities for marriage, or her husband's fertility. On the average, therefore, parity status may be looked upon as an exogenous factor. If P1 and control relatives of the same parity grouping are compared with respect to the amount of breast cancer they develop, this one exogenous factor will be held constant, and the differing results, if any, may depend largely on differences in genetic background.

Women in P1 and control groups were rated, as in table 2, on the basis of whether they had no children, 1 or 2, 3 or 4, etc. The amount of breast cancer was determined for each parity grouping in both P1 and control relatives. Only aunts and sisters could be included in the childless group, but these, as well as mothers and grandmothers, were included in groups with children. Cousins were omitted from the table, since their genetic background, on the whole, is more like that of the general population than the other 4 relationships are.

Women in the nulliparous group of P1 were tested for similarity of age distribution to those having 1 and 2 children; the latter, in turn, were tested for similarity in age distribution to those with 3 and 4, and more, each group being tested for similarity to the next highest parity group. There was no significant difference between any of the groups and the one immediately following, so that the difference in percentage of breast cancer cannot be attributed to difference in age grouping.

The different parity groups in the controls were also tested for similarity of age distribution, and no significant difference was found in the age of a group and its next highest parity group. The corresponding parity groups of P1 and the controls were tested for similarity of age distribution and no significant difference was found. Again, the difference in per-

centage of breast cancers found in P1 and the controls cannot be ascribed to any age difference in the two groups.

Not only was age distribution tested, but the similarity in decades of death was also tested. Since percentages are used here and since percentages of deaths, which are caused by breast cancer, change from decade to decade, the period of death of these relatives was also considered. There was no significant difference between P1 and controls when the relatives were tested separately for period of death distribution. The relatives were further tested for the factors of age distribution, decade of death, and parity rating, considered at the same time, and again the P1 and control relatives did not differ significantly, except in the excess of breast cancer which the P1 relatives showed in any age, parity group, and decade of death when compared with the control relatives.

Table 4 shows the effects of both the genetic and nongenetic factor of parity status. As one reads from left to right of the table, the percentage of breast cancer lessens with increasing parity ratings in both P1 and control relatives. Thus the genetic background is not enough to make all these women with different numbers of children develop breast cancer to the same extent. The same is true for the control relatives; with increasing parity, the percentage of cancer lessens. But when women from corresponding parity ratings are compared, it is at once obvious that P1 relatives have significantly more breast cancer in every parity group than control relatives. Furthermore, the highest percentage in the nulliparae in the control group is lower than the lowest percentage in the P1 relatives with 7 and 8 children. The percentages are 2.3 to 9.4 times as high in the P1 relatives as they are in the controls. The X^2 value in testing for difference between the P1 and the control relatives in corresponding parity groups is always significant at the 5 percent level, and in 4 instances significant at the 1 percent level. In comparing the P1 relatives with no children and those with 9 or more, the difference was significant ($X^2 = 7.53$), but in comparing the control relatives with no children with the P1 relatives with 9 or more, the difference was not significant ($X^2 = 0.46$). In this sample of the population there can be little doubt that the genetic background is as important as the one exogenous factor so far proved to be linked with breast cancer, namely, parity. Thus Woolf's (*4*) statement that though there is a genetic factor operative in human breast cancer, it is not as important quantitatively, as the exogenous factors must be re-examined in the light of this evidence. Incidentally, Woolf offered no support for this statement.

The totals are compared for P1 and the controls, though the comparison is of little value unless the two groups of relatives have the same proportion in each parity group. To make them comparable, the number of persons in each parity group in the controls was made the same as in P1, and the proportionate number of breast cancers which would be expected in each group was calculated. The new totals are in parentheses. There would have been 44.5 breast cancers in place of 48 in the controls, if the latter relatives had had the same parity distribution as P1.

TABLE 4.—Number and percentage of breast cancers in deceased female relatives of P1 and controls correlated with number of their children

Relatives minus cousins	Number of children							
	0	1, 2	3, 4	5, 6	7, 8	9+	Total	
P1	135	241	280	239	156	151	1202	
Breast cancers	23	17	16	13	8	10	87	
Percent of breast cancers	17. 0	7. 0	5. 7	5. 4	5. 1	6. 6	7. 2	
Controls	241	438	542	413	292	304	2230	
Breast cancers	12	13	12	4	5	2	48	(44. 5)
Percent of breast cancers	5. 0	3. 0	2. 2	1. 0	1. 7	0. 7	2. 2	(2. 0)
P is times greater than controls	3. 4	2. 3	2. 6	5. 4	3. 0	9. 4	3. 3	(3. 6)
$X^2 =$	14. 79	6. 25	6. 97	12. 05	4. 29	13. 87	58. 44	

The greatest effect of the parity factor is found in those women who had no children, both in P1 and control relatives. The greatest change occurred between women with no children and those with 1 and 2. An even greater change in percentage is noted in the nulliparous woman as one changes from close relatives of probands with breast cancer to close relatives of probands without breast cancer, though other relatives may have had breast cancer.

The same result is noted in the living relatives minus cousins of P1 and the controls (table 5). Nulliparae had the highest percentage of breast cancer in both P1 and controls, but the lowest percentage in P1 relatives was still higher than the highest in the controls. This again indicates the probable influence of genetic background. Although the differences between P1 and control relatives are not always significant in the individual parity groups, the difference for the total is significant.

TABLE 5.—Number and percentage of breast cancers in living female relatives of P1 and controls correlated with number of their children

Relatives minus cousins	Number of children					
	0	1, 2	3–6	7+	Total	
P1	133	228	233	79	673	
Breast cancers	5	6	7	1	19	
Percent of breast cancers	3. 8	2. 6	3. 0	1. 3	2. 8	
Controls	265	354	451	153	1223	
Breast cancers	3	1	1	1	6	(6. 3)
Percent of breast cancers	1. 1	0. 3	0. 2	0. 7	0. 5	(0. 5)
P is times greater than controls	3. 5	8. 7	15. 0	1. 9	5. 6	(5. 6)
$X^2 =$	3. 02	6. 40	10. 46	0. 23	18. 17	

In table 5, the various parity groups in P1 were tested for similarity of age distribution; this was done for the control groups also. The corresponding groups with the same parity rating in P1 and controls were likewise tested for similarity of distribution; in no instance were the differences significant.

From the data in tables 4 and 5, one cannot estimate quantitatively the relative status of genetic factors and the one exogenous factor studied, namely, parity. The relative importance of the two might well differ in different populations. The data presented here indicate that the genetic factors are sufficient to cause almost three times as much breast cancer among nulliparous women who are sisters or aunts of women with breast cancer as among nulliparous women from the general population in the control samples. The percentage of breast cancer among the P1 nulliparous women in table 4 (17%) was 2.1 times that of the nulliparous women (6.7%) in the Franklin County survey. Many of the latter women may have been sisters or aunts of women with breast cancer, but this fact was unknown. They are women from the general population in contrast to women who are close relatives of women with breast cancers. The drop from 17 to 7 percent in P1 relatives as one goes from women with no children to those with 1 and 2 in table 4 is not as great as the drop from 17 to 5 percent as one goes from relatives of breast-cancer probands to the controls. The same is true in table 5 in which the living relatives are listed. These data strongly suggest that the genetic factor in these familes is at least equal in importance to the parity factor.

The questions centering around the *primary* factors involved in the parity status are not answered here. For example, it may not be the actual childbearing, but the amount of nursing the woman does that offers the protection against breast cancer. To answer this, one would have to compare women of comparable age groups who have had the same number of pregnancies, but who differ from each other in that one group has nursed their children, while the other has not. Further division of the nursing group might have to be made of those who nursed (1) perhaps less than 6 months, (2) from 6 months to a year, and (3) more than a year. The responsible factor might not be the amount of nursing, but the suppression during pregnancy of the cellular changes in the breast epithelium which accompany the menstrual cycle each month. In this case, the same amount of immunity would be conferred by each full-term pregnancy, regardless of whether the children were nursed or not. Other physiological conditions associated with childbearing might be the basis of the protection against breast cancer.

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Announcements

Three United States Medical Journals Honored

The American Medical Writers' Association, at their 16th Annual Meeting in St. Louis, on October 2, presented their 1959 Honor Awards for Distinguished Service in Medical Journalism to the following publications:

1. *The Journal of the Albert Einstein Medical Center*, Philadelphia, Pennsylvania—an award for general periodicals with a circulation of less than 3,000.
2. *The Journal of the National Cancer Institute*, National Institutes of Health, Bethesda, Maryland—an award for medical periodicals devoted to some specialty in medicine or medical science.
3. *What's New*, published monthly by the Abbott Laboratories, North Chicago, Illinois—an award for medical periodicals of pharmaceutical, publishing, or related companies, designed for free national or regional interstate distribution, and mainly featuring special scientific articles, reviews, and abstracts of papers.

Dr. Morris Fishbein, president of the Association, made the presentations, which consisted of a plaque given to the respective editors. The awards, akin to the Pulitzer prizes in Journalism for newspapers, are presented annually "for accuracy, clarity, conciseness, and newness of information in articles, editorial, and other material; for excellence of design, printing, and illustrations, and for distinguished service to the medical profession." The awards were initiated in 1953, at the suggestion of, and by a grant from, Dr. Harold Swanberg, secretary of the American Medical Writers' Association.

NCI Monographs on Cancer Research

Symposia, proceedings of conferences, and, in special instances, long manuscripts or a series of manuscripts by the same author, which were formerly published in the *Journal of the National Cancer Institute*, will now be considered for publication as separate monographs.

The Board of Editors of the *Journal of the National Cancer Institute* will continue to receive and review these symposia, proceedings of conferences, and manuscripts before acceptance. The Board reserves the right to determine which manuscripts will be considered for separate publications. However, no manuscript will be published as a separate monograph without consultation and agreement of the author.

The separate monographs will be printed in the format and style of the *Journal*. These and other selected scientific publications of the National Cancer Institute will be indexed in the *Journal of the National Cancer Institute* and *Index Medicus*.

NCI Monograph No. 1—a group of papers by Dr. Hadley Kirkman, of the Stanford University School of Medicine, on "Estrogen-Induced Tumors of the Kidney in the Syrian Hamster"—is the first of the monographs. Early in 1960, a second monograph will appear entitled "Symposium on Normal and Abnormal Differentiation and Development," edited by Dr. Nathan Kaliss, of the Roscoe B. Jackson Memorial Laboratory.

Symposium on Fundamental Cancer Research

"Cell Physiology of Neoplasia" will be the topic for the Fourteenth Annual Symposium on Fundamental Cancer Research, to be held on February 25, 26, and 27, 1960, at The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston.

Further information may be obtained by writing to the Editorial Office, The University of Texas M. D. Anderson Hospital, Texas Medical Center, Houston 25, Texas.

Erratum

Volume 23, Number 3, September 1959

Page 529, line 5, in the Summary: In the paper entitled "Effect on Mice of Oral Painting of Cigarette-Smoke Condensate," by DiPaolo, *for* "No bladder tumors" *read* "One bladder tumor."

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